



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 38/17, A61P 9/00, 37/00	A2	(11) International Publication Number: WO 00/48625 (43) International Publication Date: 24 August 2000 (24.08.00)
(21) International Application Number: PCT/US00/04161 (22) International Filing Date: 17 February 2000 (17.02.00) (30) Priority Data: 09/253,604 19 February 1999 (19.02.99) US 09/444,794 22 November 1999 (22.11.99) US (71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). (72) Inventors: SHEPPARD, Paul, O.; 13532 278th Drive North-east, Granite Falls, WA 98252 (US). LASSER, Gerald, W.; 1611 S. Lake Stickney Drive, Lynnwood, WA 98037 (US). BISHOP, Paul, D.; 28425 S.E. 8th Street, Fall City, WA 98024 (US). (74) Agent: LINGENFELTER, Susan, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: INHIBITORS FOR USE IN HEMOSTASIS AND IMMUNE FUNCTION (57) Abstract The present invention relates to polynucleotide and polypeptide molecules for use as inhibitors in hemostasis and immune function. Such inhibitors are members of the family of proteins bearing a collagen-like domain and a globular domain. The inhibitors are useful for promoting blood flow in the vasculature by reducing thrombogenic and complement activity. The inhibitors are also useful for pacify collagenous surfaces and modulating wound healing.		

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Description

5 INHIBITORS FOR USE IN HEMOSTASIS AND IMMUNE FUNCTION

BACKGROUND OF THE INVENTION

10 Injury to the blood vessels sets in motion a series of events to repair the damage and control release of blood from the vessel. This process is known as hemostasis. Platelets play an early role in hemostasis by forming a thrombus or plug to temporarily repair the vessel damage. Platelets normally do not interact with
15 the endothelium lining the vessel walls, but injury to blood vessels, through accident or during surgical procedures, may disrupt endothelial cells. Depending on the extent of the injury, various subendothelial elements such as collagens, elastic lamina or smooth muscle cells
20 with associated fibrillar collagens will be exposed to the flowing blood.

 When the subendothelium is exposed following vessel injury, platelets moving in the local blood flow interact with exposed subendothelium matrix containing
25 collagen and are slowed down. Further interaction between receptors on the platelet surface and the exposed collagen layer leads to platelet binding and activation resulting in the arrest of local blood flow. The bound platelets are activated and form aggregates with platelets in the
30 passing blood flow through the formation of fibrinogen-interplatelet bridges (Moroi and Jung, Frontiers in Bioscience 3:719-28, 1998; Barnes et al., Atherosclerosis XI, Jacotot et al., eds., Elsevier Science, pp. 299-306, 1998 and Barnes et al., Curr. Opin. Hematol. 5:314-20,
35 1998).

 The hemostatic response is graded and dependent on the degree of injury to the blood vessel, the specific blood vessels constituents exposed and the blood flow

conditions in the injured area (Rand et al., Thrombosis and Haemostasis 78:445-50, 1997). Exposure of the subendothelium matrix (type VI collagen and von Willebrand factor), such as during mild vascular injury, promotes a low degree of adhesion and aggregation in areas with low blood flow conditions. Injuries that result in a greater degree of vascular trauma and exposure of additional vascular constituents, such as the internal elastic lamina and elastin-associated microfibrils, will stimulate the formation of stronger platelet aggregates. Severe vascular trauma, exposing fibril collagens, provokes a thrombotic platelet response, which protects the victim from excessive loss of blood (Rand et al., ibid.).

Inhibitors of hemostasis would be useful for to increase blood flow following vascular injury and to pacify collagenous surfaces.

Complement factor Clq consists of six copies of three related polypeptides (A, B and C chains), with each polypeptide being about 225 amino acids long with a near amino-terminal collagen domain and a carboxy-terminal globular region. Six triple helical regions are formed by the collagen domains of the six A, six B and six C chains, forming a central region and six stalks. A globular head portion is formed by association of the globular carboxy terminal domain of an A, a B and a C chain. Clq is therefore composed of six globular heads linked via six collagen-like stalks to a central fibril region. Sellar et al., Biochem. J. 274: 481-90, 1991. This configuration is often referred to as a bouquet of flowers. Acrp30 has a similar bouquet structure formed from a single type of polypeptide chain.

Clq has been found to stimulate defense mechanisms as well as trigger the generation of toxic oxygen species that can cause tissue damage (Tenner, Behring Inst. Mitt. 93:241-53, 1993). Clq binding sites are found on platelets. Additionally complement and Clq

play a role in inflammation. The complement activation is initiated by binding of C1q to immunoglobulins

Inhibitors of C1q and the complement pathway would be useful for anti-inflammatory applications, inhibition of complement activation and thrombotic activity.

The present invention provides such polypeptides for these and other uses that should be apparent to those skilled in the art from the teachings herein.

10

SUMMARY OF THE INVENTION

Within one aspect the invention provides a method of promoting blood flow within the vasculature of a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; in a pharmaceutically acceptable vehicle; whereby said adipocyte complement related protein reduces thrombogenic and complement activity within said vasculature. Within one embodiment the adipocyte complement related protein comprises a polypeptide comprising a sequence of amino acid residues that is at least 75% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2, wherein said sequence comprises: Gly-Xaa-Xaa or Gly-Xaa-Pro repeats forming a collagen domain, wherein Xaa is any amino acid, and a carboxy-terminal globular portion. Within a related embodiment the polypeptide comprises a sequence of amino acid residues that is at least 90% identical in amino acid sequence to residues 22-281 of SEQ ID NO:2. Within another embodiment the polypeptide comprises an amino acid sequence that is at least 90% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2. Within yet another embodiment any differences between said polypeptide and SEQ ID NO:2 are due to conservative amino acid substitutions. Within another embodiment the collagen domain consists of 13 Gly-Xaa-Xaa repeats and 1

Gly-Xaa-Pro repeat. Within yet another embodiment the globular domain consists of ten beta sheets. Within a related embodiment the beta sheets are associated with amino acid residues corresponding to 147-151, 170-172, 5 178-181, 191-203, 207-214, 219-225, 227-239, 244-250, and 269-274 of SEQ ID NO:2. Within yet another embodiment the polypeptide comprises residues 1-281 of SEQ ID NO:2 or residues 1-281 of SEQ ID NO:44.

The invention also provided the polypeptide is 10 complexed to a second polypeptide to form a oligomer. Within one embodiment the polypeptides are complexed by intermolecular disulfide bonds. Within another embodiment the oligomer is a trimer. Within yet another embodiment the oligomer is a hexamer. Within yet another embodiment 15 the multimer is an 18mer.

Within another embodiment the polypeptide reduces thrombogenic and complement activity by inhibition of the complement pathway and inhibition collagen-mediated platelet adhesion, activation or aggregation. Within 20 another embodiment polypeptide is administered prior to, during or following an acute vascular injury in said mammal. Within yet another embodiment the injury is due to vascular reconstruction. Within a related embodiment the vascular reconstruction comprises angioplasty, 25 coronary artery bypass graft, endarterectomy, microvascular repair or anastomosis of a vascular graft. Within another related embodiment the injury is due to trauma, stroke or aneurysm.

Within another aspect the invention provides a 30 method of pacifying damaged collagenous tissues within a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; whereby said protein renders the damaged collagenous tissue inert towards complement 35 activation, thrombotic activity or immune activation. Within one embodiment the damaged collagenous tissues are due to injury associated with ischemia and reperfusion.

Within another embodiment the injury comprises trauma injury ischemia, intestinal strangulation, or injury associated with pre- and post-establishment of blood flow. Within yet another embodiment the polypeptide is administered to a mammal suffering from cardiopulmonary bypass ischemia and recirculation, myocardial infarction, or post-trauma vasospasm. Within a related embodiment the post-trauma vasospasm comprises stroke, percutaneous transluminal angioplasty, endarterectomy, accidental vascular trauma or surgical-induced vascular trauma.

Within yet another aspect the invention provides a method of pacifying the surface of a prosthetic biomaterial for use in association with a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; whereby said polypeptide renders the surface of said prosthetic biomaterial inert towards complement activation, thrombotic activity or immune activation. Within one embodiment the surface of said prosthetic biomaterial is coated with collagen or collagen fragments, gelatin, fibrin or fibronectin.

Within another aspect of the invention is provided a method of mediating wound repair within a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; whereby said polypeptide enhances progression in wound healing.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a multiple alignment of and zsig37 polypeptide of the present invention and HUMUPST2_1 (Maeda et al., Biochem. Biophys. Res. Comm. 221(2): 286-9, 1996); C1QA_HUMAN (Sellar et al., Biochem. J. 274: 481-90, 1991, Reid, Biochem. J. 179: 367-71, 1979, and Reid et al., Biochem. J. 203: 559-69, 1982); HP25_TAMAS (Takamatsu et al., Mol. Cell. Biol. 13: 1516-21, 1993 and Kondo & Kondo, J. Biol. Chem. 267: 473-8, 1992); HP27_TAMAS

(Takamatsu et al. and Kondo & Kondo referenced above); and CERL_RAT (Wada & Ohtani, Brain Res. Mol. Brain Res. 9: 71-7, 1991).

Figure 2 is a matrix showing percent amino acid identity in a comparison of the six proteins shown in the multiple alignment Fig. 1.

Figure 3a shows zsig37-FITC binding to type VI collagen.

Figure 3b shows competition of unlabeled zsig37 with FITC labeled zsig37 bound to type VI collagen.

Figure 4 shows binding of complement C1q-FITC to zsig37.

Figure 5 shows inhibition of human complement activity by zsig37.

Figure 6 shows the percent aggregation of platelets by collagen in the presence of zsig37.

Figure 7 shows proliferation of SK5 fibroblasts in the presence of zsig37.

20 DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms.

25 The term "affinity tag" is used herein to denote a peptide segment that can be attached to a polypeptide to provide for purification or detection of the polypeptide or provide sites for attachment of the polypeptide to a substrate. In principal, any peptide or
30 protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase
35 (Smith and Johnson, Gene 67:31, 1988), substance P, Flag™ peptide (Hopp et al., Biotechnology 6:1204-10, 1988; available from Eastman Kodak Co., New Haven, CT),

streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial
5 suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term ``complements of a polynucleotide molecule'' is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the
10 sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term ``degenerate nucleotide sequence'' denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference
15 polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term ``isolated'', when applied to a
20 polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are
25 those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as
30 promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985).

An ``isolated'' polypeptide or protein is a
35 polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated

polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than

about 10 amino acid residues are commonly referred to as "peptides".

``Probes and/or primers'' as used herein can be RNA or DNA. DNA can be either cDNA or genomic DNA. Polynucleotide probes and primers are single or double-stranded DNA or RNA, generally synthetic oligonucleotides, but may be generated from cloned cDNA or genomic sequences or its complements. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, preferably 15 or more nt, more preferably 20-30 nt. Short polynucleotides can be used when a small region of the gene is targeted for analysis. For gross analysis of genes, a polynucleotide probe may comprise an entire exon or more. Probes can be labeled to provide a detectable signal, such as with an enzyme, biotin, a radionuclide, fluorophore, chemiluminescer, paramagnetic particle and the like, which are commercially available from many sources, such as Molecular Probes, Inc., Eugene, OR, and Amersham Corp., Arlington Heights, IL, using techniques that are well known in the art.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as ``about'' X or ``approximately'' X, the stated value of X will be understood to be accurate to $\pm 10\%$.

The present invention was based in part upon the discovery that a novel adipocyte complement related protein homolog inhibits collagen-mediated platelet activation and the complement pathway including C1q. This protein was designated zsig37 and is fully described in the commonly assigned published PCT patent application WO 99/04000.

The zsig37 nucleotide sequence (SEQ ID NO:1) encodes a polypeptide (SEQ ID NO:2) having an amino-

terminal signal sequence, an adjacent N-terminal region of non-homology, a truncated collagen domain composed of Gly-Xaa-Xaa or Gly-Xaa-Pro repeats and a carboxy-terminal globular portion. The novel polynucleotide sequence also
5 contains a long 3' untranslated region. The general polypeptide structure set forth above is shared by Acrp30 and HUMUPST2_1, except that the collagen-like domain of each of those proteins is longer than that of zsig37 polypeptides. Also, the HUMUPST2_1 DNA sequence is
10 characterized by a long 3' untranslated region. Moreover, Acrp30 and all of the sequences aligned in Fig. 1, with the exception of CERL_RAT, share a conserved cysteine residue at position 187 of zsig37 polypeptide as shown in Fig. 1 and SEQ ID NO: 2. Also, the zsig37 polypeptides of
15 the present invention include a putative N-linked glycosylation site at amino acid 93 (Asn) of SEQ ID NO: 2.

Analysis of the tissue distribution of the mRNA corresponding to zsig37 showed that expression was highest in heart and placenta, with relatively less intense
20 signals in kidney, ovary, adrenal gland and skeletal muscle and lower signals in a wide variety of other tissues present on the Northern blot.

A homolog relationship with adipocyte complement related protein Acrp30 (SEQ ID NO: 3) and adipocyte
25 secreted protein apM1 (HUMUPST2_1 in Figs. 1 and 2) was established for zsig37. Somewhat more distant homology was also identified to complement component C1q A chain, two factors observed in the active state of hibernating Siberian woodchucks (HP25_TAMAS and HP27_TAMAS) and a rat
30 brain protein (CERL_RAT), as shown in Figs. 1 and 2.

The nucleotide sequence of zsig37 is described in SEQ ID NO: 1, and its deduced amino acid sequence is described in SEQ ID NO: 2. A degenerate nucleotide
35 sequence encoding the polypeptide of SEQ ID NO:2 is provided in SEQ ID NO:23. As described generally above, the zsig37 polypeptide includes a signal sequence, ranging from amino acid 1 (Met) to amino acid residue 21 (Gly).

An alternative signal sequence ranges from amino acid 1 (Met) to amino acid 25 (Ser). The mature polypeptide therefore ranges from amino acid 22 (Leu) or 26 (Arg) to amino acid 281 (Pro). Within the mature polypeptide, an N-terminal region of no known homology is found, ranging between amino acid residue 22 (Leu) and 98 (Lys). In addition, a truncated collagen domain is found between amino acid 99 (Gly) and 140 (Arg). In the truncated collagen domain, 1 perfect Gly-Xaa-Pro and 13 imperfect Gly-Xaa-Xaa repeats are observed. In contrast, Acrp30 contains 22 perfect or imperfect repeats. The zsig37 polypeptide also includes a carboxy-terminal globular domain, ranging from about amino acid 141 (Cys) to 281 (Pro). Zsig37 polypeptide, HUMUPST2_1 and Acrp30 appear to be homologous within the collagen domain and in the globular domain, but not in the N-terminal portion of the mature polypeptide.

The globular Clq domain of ACRP30 has been determined to have a 10 beta strand "jelly roll" topology (Shapiro and Scherer, Curr. Biol. 8:335-8, 1998) that shows significant structural homology to the TNF family and the zsig37 sequence as represented by SEQ ID NO:2 contains all 10 beta-strands of this structure (amino acid residues 147-151, 170-172, 178-181, 185-188, 191-203, 207-214, 219-225, 227-238, 244-250, and 269-274 of SEQ ID NO:2). These strands have been designated "A", "A'", "B", "B'", "C", "D", "E", "F", "G" and "H" respectively.

Zsig37 has two receptor binding loops, at amino acid residues 152-180 and 213-226. Amino acid residues 191 (Gly), 193 (Tyr), 238 (Leu) and 272 (Gly) appear to be conserved across the superfamily including CD40, TNF α , TNF β , ACRP30 and zsig37.

Another aspect of the present invention includes use of zsig37 polypeptide fragments as inhibitors of hemostasis and immune functions. Preferred fragments include the collagen-like domain of zsig37 polypeptides,

ranging from amino acid 99 (Gly) to amino acid 140 (Arg) of SEQ ID NO:2, a portion of the zsig37 polypeptide containing the collagen-like domain or a portion of the collagen-like domain capable of dimerization or oligomerization. Other preferred fragments include the globular domain of zsig37 polypeptides, ranging from amino acid 140 (Arg) or 141 (Cys) to 281 (Pro) of SEQ ID NO: 2, a portion of the zsig37 polypeptide containing the globular-like domain or an active portion of the globular-like domain. Another zsig37 polypeptide fragment of the present invention include both the collagen-like domain and the globular domain ranging from amino acid residue 99 (Gly) to 281 (Pro) of SEQ ID NO: 2. These fragments are particularly useful in the inhibition of collagen-mediated platelet activation and inhibition of complement and C1q.

The present invention also provides use of zsig37 fusion proteins. For example, fusion proteins of the present invention encompass (1) a polypeptide selected from the group comprising: (a) polypeptide molecules comprising a sequence of amino acid residues as shown in SEQ ID NO: 2 from amino acid residue 1 (Met), 22 (Leu) or 26 (Arg) to amino acid residue 281 (Pro); (b) polypeptide molecules ranging from amino acid 99 (Gly) to amino acid 140 (Arg) of SEQ ID NO: 2, a portion of the zsig37 polypeptide containing the collagen-like domain or a portion of the collagen-like domain capable of dimerization or oligomerization; (c) polypeptide molecules ranging from amino acid 140 (Arg) or 141 (Cys) to 281 (Pro) of SEQ ID NO: 2, a portion of the zsig37 polypeptide containing the globular-like domain or an active portion of the globular-like domain; or (d) polypeptide molecules ranging from amino acid 99 (Gly) to 281 (Pro), a portion of the zsig37 polypeptide including the collagen-like domain and the globular domain; and (2) another polypeptide. The other polypeptide may be alternative or additional globular domain, an alternative or additional

collagen-like domain, a signal peptide to facilitate secretion of the fusion protein or the like.

Also useful within the methods of the invention are zsig37 agonists and antagonists. Methods of identifying antagonists are known in the art. For example, antagonists of the zsig37 polypeptide can be identified by providing cells responsive to a zsig37 polypeptide, culturing a first portion of the cells in the presence of zsig37 polypeptide, culturing a second portion of the cells in the presence of the zsig37 polypeptide and a test compound, and detecting a decrease in a cellular response of the second portion of the cells as compared to the first portion of the cells. In addition to those assays disclosed herein, samples can be tested for inhibition of zsig37 activity within a variety of assays designed to measure receptor binding or the stimulation/inhibition of zsig37-dependent cellular responses. For example, zsig37-responsive cell lines can be transfected with a reporter gene construct that is responsive to a zsig37-stimulated cellular pathway. Reporter gene constructs of this type are known in the art, and will generally comprise a zsig37-DNA response element operably linked to a gene encoding an assayable protein, such as luciferase. DNA response elements can include, but are not limited to, cyclic AMP response elements (CRE), hormone response elements (HRE), insulin response element (IRE) (Nasrin et al., Proc. Natl. Acad. Sci. USA 87:5273-7, 1990) and serum response elements (SRE) (Shaw et al. Cell 56: 563-72, 1989). Cyclic AMP response elements are reviewed in Roestler et al., J. Biol. Chem. 263 (19):9063-6, 1988 and Habener, Molec. Endocrinol. 4 (8):1087-94, 1990. Hormone response elements are reviewed in Beato, Cell 56:335-44; 1989. Candidate compounds, solutions, mixtures or extracts are tested for the ability to inhibit the activity of zsig37 on the target cells as evidenced by a decrease in zsig37 stimulation of reporter gene expression. Assays of this

type will detect compounds that directly block zsig37 binding to cell-surface receptors, as well as compounds that block processes in the cellular pathway subsequent to receptor-ligand binding. In the alternative, compounds or
5 other samples can be tested for direct blocking of zsig37 binding to receptor using zsig37 tagged with a detectable label (e.g., ¹²⁵I, biotin, horseradish peroxidase, FITC, and the like). Within assays of this type, the ability of a test sample to inhibit the binding of labeled zsig37 to
10 the receptor is indicative of inhibitory activity, which can be confirmed through secondary assays. Receptors used within binding assays may be cellular receptors or isolated, immobilized receptors.

Also useful within the methods of the invention
15 are antibodies that specifically bind to zsig37 polypeptide epitopes, peptides or polypeptides. Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition,
20 Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982).

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from
25 inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, hamsters, guinea pigs and rats as well as transgenic animals such as transgenic sheep, cows, goats or pigs. Antibodies may also be expressed in yeast and fungi in
30 modified forms as well as in mammalian and insect cells. The zsig37 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal or elicit an immune response. Suitable antigens would include the zsig37 polypeptide encoded by SEQ ID NO:2 from amino acid
35 residue 22-281 of SEQ ID NO:2, from amino acid residue 26-281 of SEQ ID NO:2, or a contiguous 9-281 amino acid residue fragment thereof. The immunogenicity of a zsig37

polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as
5 fusions of zsig37 or a portion thereof with an immunoglobulin polypeptide or with an affinity tag. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked
10 to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal
15 antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab')₂ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-
20 binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by
25 replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies,
30 biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to zsig37 protein or peptide, and
35 selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled zsig37 protein or peptide).

Antibodies are defined to be specifically binding if: 1) they exhibit a threshold level of binding activity, and/or 2) they do not significantly cross-react with related polypeptide molecules. First, antibodies
5 herein specifically bind if they bind to a zsig37 polypeptide, peptide or epitope with a binding affinity (K_a) of 10^6 mol^{-1} or greater, preferably 10^7 mol^{-1} or greater, more preferably 10^8 mol^{-1} or greater, and most preferably 10^9 mol^{-1} or greater. The binding affinity of
10 an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, Ann. NY Acad. Sci. 51: 660-672, 1949).

Second, antibodies specifically bind if they do not significantly cross-react with related polypeptides.
15 Antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect zsig37 polypeptide but not known related polypeptides using a standard Western blot analysis (Ausubel et al., *ibid.*). Examples of known related polypeptides include other
20 members of a protein family such as Acrp30 (SEQ ID NO:3), the polypeptides shown in alignment Fig. 1 and the like. They could also include, if desired, orthologs and mutant human zsig37 polypeptides. Moreover, antibodies may be ``screened against'' known related polypeptides to isolate
25 a population that specifically binds to the inventive polypeptides. For example, antibodies raised to human zsig37 polypeptides are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to human zsig37 polypeptides will flow through the matrix under the
30 proper buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to closely related polypeptides (Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; Current Protocols in Immunology,
35 Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art (see,

Fundamental Immunology, Paul (eds.), Raven Press, 1993; Getzoff et al., Adv. in Immunol. 43: 1-98, 1988; Monoclonal Antibodies: Principles and Practice, Goding, J.W. (eds.), Academic Press Ltd., 1996; Benjamin et al., 5 Ann. Rev. Immunol. 2: 67-101, 1984). Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition 10 assay, and sandwich assay.

The effect of zsig37 polypeptides, fragments, fusions, agonists or antagonists on hemostasis, in particular platelet adhesion and activation leading to platelet aggregation can be determined by using methods 15 and assays provided herein and those known in the art. Collagen is a potent inducer of platelet aggregation. This poses risks to patients recovering from vascular injuries. Inhibitors of collagen-induced platelet aggregation would be useful for such purposes. Zsig37 was 20 found to bind to fibronectin and type I, II, III, V and VI collagens. In particular, zsig37 binds to specific domains on collagen VI in a concentration dependent manner. Zsig37 was also found to inhibit collagen-mediated platelet activation. Zsig37-induced inhibition 25 was selective for collagen activation, zsig37 had no effect on platelets activated by known platelet activators ADP or thrombin. These results are described in more detail below in the Example section below. It is anticipated that zsig37 polypeptides, fragments, fusions, 30 agonists or antagonists will be useful for blocking the binding of platelets to collagen-coated surfaces and reducing associated collagen-induced platelet aggregation.

C1q is a component of the complement pathway and has been found to stimulate defense mechanisms as well as 35 trigger the generation of toxic oxygen species that can cause tissue damage (Tenner, Behring Inst. Mitt. 93:241-53, 1993). C1q binding sites are found on platelets.

C1q, independent of an immune binding partner, has been found to inhibit platelet aggregation but not platelet adhesion or shape change. The amino terminal region of C1q shares homology with collagen (Peerschke and Ghebrehiwet, J. Immunol. 145:2984-88, 1990). Zsig37 binds to complement C1q in a concentration dependent manner. Zsig37 was found to be effective in inhibiting the complement pathway including C1q with both sensitized and unsensitized sheep erythrocytes.

Zsig37 polypeptides, fragments, fusion proteins, antibodies, agonists or antagonists of the present invention can be used in methods for promoting blood flow within the vasculature of a mammal by reducing the number of platelets that adhere and are activated and the size of platelet aggregates. Such methods would comprise administration of a therapeutically effective amount of zsig37 polypeptides, fragments, fusions, antibodies, agonists or antagonists to a mammal in need of such treatment, whereby zsig37 reduces thrombogenic and complement activity within the vasculature of the mammal. As is described below, zsig37 polypeptides inhibit collagen-mediated platelet activation and inactivate fibronectin and type I, II, III, V and VI collagens through binding. Zsig37 administration reduces thrombogenic activity at the site of vascular injury by reducing the modes for platelet adhesion, activation and aggregation. Zsig37 also inhibits the complement pathway and C1q as is described below, thus reducing complement activity within the vasculature. Zsig37 polypeptides, fragments, fusions, antibodies, agonists or antagonists used in such methods can be administered prior to, during or following an acute vascular injury in the mammal.

In a preferred method, the vascular injury is due to vascular reconstruction, including but not limited to, angioplasty, endarterectomy, coronary artery bypass graft, microvascular repair or anastomosis of a vascular graft. Also contemplated are vascular injuries due to

trauma, stroke or aneurysm. In other preferred methods the vascular injury is due to plaque rupture, degradation of the vasculature, complications associated with diabetes and atherosclerosis. Plaque rupture in the coronary
5 artery induces heart attack and in the cerebral artery induces stroke. Use of zsig37 polypeptides, fragments, fusion proteins, antibodies, agonists or antagonists in such methods would also be useful for ameliorating whole system diseases of the vasculature associated with the
10 immune system, such as disseminated intravascular coagulation (DIC) and SIDs. Additionally the complement inhibiting activity would be useful for treating non-vasculature immune diseases such as arteriolosclerosis.

A correlation has been found between the
15 presence of C1q in localized ischemic myocardium and the accumulation of leukocytes following coronary occlusion and reperfusion. Release of cellular components following tissue damage triggers complement activation which results in toxic oxygen products that may be the primary cause of
20 myocardial damage (Rossen et al., Circ. Res. 62:572-84, 1998 and Tenner, ibid.). Blocking the complement pathway was found to protect ischemic myocardium from reperfusion injury (Buerke et al., J. Pharm. Exp. Therp. 286:429-38, 1998). The complement inhibition and C1q binding activity
25 of zsig37 polypeptides would be useful for such purposes.

The collagen and C1q binding capabilities of zsig37 would be useful to pacify damaged collagenous tissues preventing platelet adhesion, activation or aggregation, and the activation of inflammatory processes
30 which lead to the release of toxic oxygen products. By rendering the exposed tissue inert towards such processes as complement activity, thrombotic activity and immune activation, zsig37 polypeptides, fragments, fusions, antibodies, agonists or antagonists would be useful in
35 reducing the injurious effects of ischemia and reperfusion. In particular, such injuries would include trauma injury ischemia, intestinal strangulation, and

injury associated with pre- and post-establishment of blood flow. Zsig37 would be useful in the treatment of cardiopulmonary bypass ischemia and recesitation, myocardial infarction and post trauma vasospasm, such as
5 stroke or percutaneous transluminal angioplasty as well as accidental or surgical-induced vascular trauma.

Zsig37 polypeptides, fragments, fusions, antibodies, agonists or antagonists would also be useful to pacify prosthetic biomaterials and surgical equipment
10 to render the surface of the materials inert towards complement activation, thrombotic activity or immune activation. Such materials include, but are not limited to, collagen or collagen fragment-coated biomaterials, gelatin-coated biomaterials, fibrin-coated biomaterials,
15 fibronectin-coated biomaterials, heparin-coated biomaterials, collagen and gel-coated stents, arterial grafts, synthetic heart valves, artificial organs or any prosthetic application exposed to blood that will bind zsig37 at greater than 1×10^8 . Coating such materials can
20 be done using methods known in the art, see for example, Rubens, US Patent No. 5,272,074.

Complement and C1q play a role in inflammation. The complement activation is initiated by binding of C1q to immunoglobulins (Johnston, Pediatr. Infect. Dis. J.
25 12:933-41, 1993; Ward and Ghetie, Therap. Immunol. 2:77-94, 1995). Inhibitors of C1q and complement would be useful as anti-inflammatory agents. Such application can be made to prevent infection. Additionally, such inhibitors can be administrated to an individual suffering
30 from inflammation mediated by complement activation and binding of immune complexes to C1q. Zsig37 polypeptides, fragments, fusion proteins, antibodies, agonists or antagonists would be useful in methods of mediating wound repair, enhancing progression in wound healing by
35 overcoming impaired wound healing. Progression in wound healing would include, for example, such elements as a

reduction in inflammation, fibroblasts recruitment, wound retraction and reduction in infection.

Ability of tumor cells to bind to collagen may contribute to the metastasis of tumors. Inhibitors of collagen binding are also useful for mediating the adhesive interactions and metastatic spread of tumors (Noeske-Jungbult et al., US Patent No. 5,723,312).

Zsig37 was found to induce vasodilatation in norepinephrin-contracted aortic rings using the procedures of Dainty et al., J. Pharmacol. 100:767, 1990 and Rhee et al., Neurotox. 16:179, 1995, as is described below in greater detail.

Platelet adhesion, activation and aggregation can be evaluated using methods described herein or known in the art, such as the platelet aggregation assay (Chiang et al., Thrombosis Res. 37:605-12, 1985) and platelet adhesion assays (Peerschke and Ghebrehiwet, J. Immunol. 144:221-25, 1990). Inhibition of C1q and the complement pathway can be determined using methods disclosed herein or known in the art, such as described in Suba and Csako, J. Immunol. 117:304-9, 1976. Assays for platelet adhesion to collagen and inhibition of collagen-induced platelet aggregation can be measured using methods described in Keller et al., J. Biol. Chem. 268:5450-6, 1993; Waxman and Connolly, J. Biol. Chem. 268:5445-9, 1993; Noeske-Jungblut et al., J. Biol. Chem. 269:5050-3 or 1994 Deckmyn et al., Blood 85:712-9, 1995.

Various *in vitro* and *in vivo* models are available for assessing the effects of zsig37 polypeptides, fragments, fusion proteins, antibodies, agonists and antagonists on ischemia and reperfusion injury. See for example, Shandelya et al., Circulation 88:2812-26, 1993; Weisman et al., Science 249:146-151, 1991; Buerke et al., Circulation 91:393-402, 1995; Horstick et al., Circulation 95:701-8, 1997 and Burke et al., J. Phar. Exp. Therp. 286:429-38, 1998. An *ex vivo* hamster platelet aggregation assay is described by Deckmyn

et al., ibid. Bleeding times in hamsters and baboons can be measured following injection of zsig37 polypeptides using the model described by Deckmyn et al., ibid. The formation of thrombus in response to administration of proteins of the present invention can be measured using the hamster femoral vein thrombosis model is provided by Deckmyn et al., ibid. Changes in platelet adhesion under flow conditions following administration of zsig37 can be measured using the method described in Harsfalvi et al., Blood 85:705-11, 1995.

Complement inhibition and wound healing can be Zsig37 polypeptides, fragments, fusion proteins, antibodies, agonists or antagonists be assayed alone or in combination with other know inhibitors of collagen-induced platelet activation and aggregation, such as palldipin, moubatin or calin, for example.

Zsig37 polypeptides, fragments, fusion proteins, antibodies, agonists or antagonists can be evaluated using methods described herein or known in the art, such as healing of dermal layers in pigs (Lynch et al., Proc. Natl. Acad. Sci. USA 84: 7696-700, 1987) and full-thickness skin wounds in genetically diabetic mice (Greenhalgh et al., Am. J. Pathol. 136: 1235-46, 1990), for example. The polypeptides of the present invention can be assayed alone or in combination with other known complement inhibitors as described above.

In addition, zsig37 polypeptides, fragments, fusions agonists or antagonists thereof may be therapeutically useful for anti-microbial applications. For example, complement component Clq plays a role in host defense against infectious agents, such as bacteria and viruses. Clq is known to exhibit several specialized functions. For example, Clq triggers the complement cascade via interaction with bound antibody or C-reactive protein (CRP). Also, Clq interacts directly with certain bacteria, RNA viruses, mycoplasma, uric acid crystals, the lipid A component of bacterial endotoxin and membranes of

certain intracellular organelles. Clq binding to the Clq receptor is believed to promote phagocytosis. Clq also appears to enhance the antibody formation aspect of the host defense system. See, for example, Johnston, Pediatr. Infect. Dis. J. 12(11): 933-41, 1993. Thus, soluble Clq-like molecules may be useful as anti-microbial agents, promoting lysis or phagocytosis of infectious agents.

The positively charged, extracellular, triple helix, collagenous domains of Clq and macrophage scavenger receptor were determined to play a role in ligand binding and were shown to have a broad binding specificity for polyanions (Acton et al., J. Biol. Chem. 268:3530-37, 1993). Lysophospholipid growth factor (lysophosphatidic acid, LPA) and other mitogenic anions localize at the site of damaged tissues and assist in wound repair. LPA exerts many biological effects including activation of platelets and up-regulation of matrix assembly. It is thought that LPA synergizes with other blood coagulation factors and mediates wound healing.

The collagenous domains of proteins such as Clq and macrophage scavenger receptor are known to bind acidic phospholipids such as LPA. A 9mer region of the collagen domain of zsig37, amino acid residues 127-135 of SEQ ID NO:2, shares sequence homology with the collagen domain found on Clq and macrophage scavenger receptor. The interaction of zsig37 polypeptides, fragments, fusions, agonists or antagonists with mitogenic anions such as LPA can be determined using assays known in the art, see for example, Acton et al., ibid. Inhibition of inflammatory processes by polypeptides and antibodies of the present invention would also be useful in preventing infection at the wound site.

For pharmaceutical use, the proteins of the present invention can be formulated with pharmaceutically acceptable carriers for parenteral, oral, nasal, rectal, topical, transdermal administration or the like, according to conventional methods. Preferably administration is

made at or near the site of vascular injury. In general, pharmaceutical formulations will include a zsig37 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton PA, 19th ed., 1995. Therapeutic doses will generally be determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art.

As used herein a "pharmaceutically effective amount" of a zsig37 polypeptide, fragment, fusion protein, agonist or antagonist is an amount sufficient to induce a desired biological result. The result can be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an effective amount of a zsig37 polypeptide is that which provides either subjective relief of symptoms or an objectively identifiable improvement as noted by the clinician or other qualified observer. Such an effective amount of a zsig37 polypeptide would provide, for example, inhibition of collagen-activated platelet activation and the complement pathway, including C1q, increase localized blood flow within the vasculature of a patient and/or reduction in injurious effects of ischemia and reperfusion. Effective amounts of the zsig37 polypeptides can vary widely depending on the disease or symptom to be treated. The amount of the polypeptide to be administered and its concentration in the formulations, depends upon the vehicle selected, route of administration, the potency of the particular polypeptide, the clinical condition of

the patient, the side effects and the stability of the compound in the formulation. Thus, the clinician will employ the appropriate preparation containing the appropriate concentration in the formulation, as well as
5 the amount of formulation administered, depending upon clinical experience with the patient in question or with similar patients. Such amounts will depend, in part, on the particular condition to be treated, age, weight, and general health of the patient, and other factors evident
10 to those skilled in the art. Typically a dose will be in the range of 0.01-100 mg/kg of subject. In applications such as balloon catheters the typical dose range would be 0.05-5 mg/kg of subject. Doses for specific compounds may be determined from *in vitro* or *ex vivo* studies in
15 combination with studies on experimental animals. Concentrations of compounds found to be effective *in vitro* or *ex vivo* provide guidance for animal studies, wherein doses are calculated to provide similar concentrations at the site of action.

20 The invention is further illustrated by the following non-limiting examples.

Example 1

Extension of EST Sequence

25

The novel zsig37 polypeptide-encoding polynucleotides of the present invention were initially identified by selecting an EST from an EST database, predicting a protein sequence based thereupon, and
30 searching known sequence databases for the secreted protein that is most homologous to predicted protein based on the EST. ESTs that potentially encode proteins having biologically interesting homology to known secreted proteins were identified for further study. A single EST
35 sequence was discovered and predicted to be homologous to adipocyte specific protein. See, for example, Scherer et al., J. Biol. Chem. 270(45): 26746-9, 1995. To identify

the corresponding cDNA, a clone considered likely to contain the entire coding sequence was used for sequencing. Using an Invitrogen S.N.A.P.TM Miniprep kit (Invitrogen, Corp., San Diego, CA) according to
5 manufacturer's instructions a 5 ml overnight culture in LB + 50 µg/ml ampicillin was prepared. The template was sequenced on an ABIPRISMTM model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, Ct.) using the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit
10 (Perkin-Elmer Corp.) according to manufacturer's instructions. Oligonucleotides ZC695 (SEQ ID NO: 5), ZC694 (SEQ ID NO: 6) to the SP6 and T7 promoters on the clone-containing vector were used as sequencing primers. Oligonucleotides ZC13210 (SEQ ID NO: 7), ZC13588 (SEQ ID
15 NO: 8), ZC13532 (SEQ ID NO: 9), ZC13641 (SEQ ID NO: 10), ZC13586 (SEQ ID NO: 11), ZC13651 (SEQ ID NO: 12), ZC13622 (SEQ ID NO: 13), ZC13625 (SEQ ID NO: 14), ZC13650 (SEQ ID NO: 15), ZC13589 (SEQ ID NO: 16), ZC13624 (SEQ ID NO: 17), ZC13531 (SEQ ID NO: 18), ZC13587 (SEQ ID NO: 19), and
20 ZC13623 (SEQ ID NO: 20) were used to complete the sequence from the clone. Sequencing reactions were carried out in a Hybaid OmniGene Temperature Cycling System (National Labnet Co., Woodbridge, NY). SEQUENCHERTM 3.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI)
25 was used for data analysis. The resulting 2769 bp sequence is disclosed in SEQ ID NO: 1. Comparison of the originally derived EST sequence with the sequence represented in SEQ ID NO: 1 showed that there was one base pair ambiguity (an unknown ``N'' residue) and no base pair
30 insertions which resulted in the identification of leucine in resolution of the ambiguity and zero frame shifts between the deduced amino acid sequences.

Northerns were performed using Human Multiple Tissue Blots from Clontech (Palo Alto, CA). A 30 base DNA probe (ZC12447; SEQ ID NO: 4) to the 5' end of the nucleotide sequence of the mature protein shown in SEQ ID NO: 1 was radioactively labeled with ^{32}P using T4 polynucleotide kinase and forward reaction buffer (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene Cloning Systems, La Jolla, CA). EXPRESSHYB (Clontech, Palo Alto, CA) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 50°C, and the blots were then washed in 2X SSC and 0.1% SDS at RT, followed by a wash in 1X SSC and 0.1% SDS at 68°C (about 5°C less than the melting point). One transcript size was observed at approximately 2.8 kb. Signal intensity was highest for heart and placenta, with relatively less intense signals in kidney, ovary, adrenal gland and skeletal muscle and lower signals in a wide variety of other tissues present on the Northern blot.

Additional Northern Blot Analysis was done using a Gut Northern Tissue Blot. The blot was prepared using mRNA from human colorectal adenocarcinoma cell line SW480 (Clontech, Palo Alto, CA), human small intestine tissue (Clontech), human stomach tissue (Clontech), human intestinal smooth muscle cell line (Hism; ATCC No. CRL-1692; American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD), normal human colon cell line (FHC; ATCC No. CRL-1831; American Type Culture Collection) and human normal fetal small intestine cell line (FHS74 Int.; ATCC No. CCL241; American Type Culture Collection).

Total RNAs were isolated from Hism, FHC and FHS74 Int. by acid guanidium method (Cheomczynski et al., Anal. Biochem. 162:156-9, 1987). The polyA⁺ RNAs were selected by eluting total RNA through a column that retains polyA⁺ RNAs (Aviv et al., Proc. Nat. Acad. Sci. 69:1408-12, 1972). 2 µg of polyA⁺ RNA from each sample was

separated out in a 1.5% agarose gel in 2.2 M formaldehyde and phosphate buffer. The RNAs were transferred onto Nytran membrane (Schleicher and Schuell, Keene, NH) in 20X SSC overnight. The blot was treated in the UV
5 Stratalinker 2400 (Stratagene, La Jolla, CA) at 0.12 Joules. The blot was then baked at 80°C for one hour.

Full length cDNA (shown in SEQ ID NO: 1) was amplified by PCR and radiolabeled with ³²P dCTP using a Rediprime pellet kit (Amersham, Arlington Heights, IL)
10 according to the manufacturer's specifications. The blot was hybridized in EXPRESSHYB (Clontech) at 56°C overnight. The blot was washed at room temperature in 2X SSC and 0.1% SDS, then in 2X SSC and 0.1% SDS at 65°C, and finally at 65°C in 0.1X SSC and 0.1% SDS. Results showed that zsig37
15 hybridized to all tissues except the human intestinal smooth muscle cell line HISM.

Example 3 Chromosomal Mapping of the Zsig37 Gene

20

The zsig37 gene was mapped to human chromosome 17, region 17q25.2, by PCR using the NIGMS Human/Rodent Somatic Cell Hybrid Mapping Panel Number 2 (National Institute of General Medical Sciences, Coriell Institute
25 of Medical Research). The panel consists of DNA isolated from 24 human/rodent somatic cell hybrids each retaining one specific human chromosome and the parental DNAs. For the mapping of the zsig37 gene, 20 µl reactions were set up in a 96-well microtiter plate (Stratagene, La Jolla,
30 CA) and used in a "RoboCycler Gradient 96" thermal cycler (Stratagene). Each of the 27 PCR reactions consisted of 2 µl 10X KlenTaq PCR reaction buffer (Clontech Laboratories, Inc., Palo Alto, CA), 1.6 µl dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1 µl sense primer (SEQ ID
35 NO: 21), 1 µl antisense primer (SEQ ID NO: 22), 2 µl RediLoad (Research Genetics, Inc.), 0.4 µl 50X Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and

ddH₂O for a total volume of 20 μ l. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cyclers conditions were as follows: an initial 1 cycle 5 minute denaturation at 95°C, 35 cycles of a 1 minute denaturation at 95°C, 1 minute annealing at 60°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

10

Example 4
Creation of mammalian expression vectors
zsig37NEE/pZP9 and zsig37CEE/pZP9

15

Two expression vectors were prepared for the zsig37 polypeptide, zSIG37NEE/pZP9 and zSIG37CEE/pZP9, wherein the constructs were designed to express a zsig37 polypeptide having a C- or N-terminal Glu-Glu tag.

20

Zsig37NEE/pZP9

A 800 bp PCR generated zsig-37 DNA fragment was created using ZC15040 (SEQ ID NO:24) and ZC15033 (SEQ ID NO:25) as PCR primers and the template described in Example 1 above. The PCR reaction was incubated at 94°C for 3 minutes, and then run for 5 cycles of 94°C for 30 seconds, 30°C for 20 seconds and 72°C for 1 minute, followed by 25 cycles at 94°C for 30 seconds, 64°C for 20 seconds and 72°C for 1 minute. A 5 minute extension at 72°C followed. The resultant PCR product was then run on a 0.9% TBE agarose gel with 1x TBE buffer. A band of the predicted size was excised and the DNA was purified from the gel with a Qiaex II[®] resin (Qiagen) according the manufacturer's instructions. The DNA was digested with the restriction enzymes Bam HI and Xba I, followed by extraction and precipitation.

25

30

35

The excised, restriction digested zsig37 DNA fragment was subcloned into plasmid NEE/pZP9 which had

been cut with the restriction enzymes Bam HI and Xba I. The zsig37NEE/pZP9 expression vector incorporates the TPA leader and attaches a Glu-Glu tag (SEQ ID NO:26) to the N-terminal of the zsig37 polypeptide-encoding polynucleotide
5 sequence. Plasmid NEE/pZP9 (deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, ATCC No. 98668) is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, a TPA leader peptide followed
10 by the sequence encoding the Glu-Glu tag (SEQ ID NO:26), multiple restriction sites for insertion of coding sequences, and a human growth hormone terminator. The plasmid also contains an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40
15 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

zsig376CEE/pZP9

A 866 bp PCR generated zsig37 DNA fragment was
20 created in accordance with the procedure set forth above using ZC15721 (SEQ ID NO:27) and ZC15035 (SEQ ID NO:28) as PCR primers. The purified PCR fragment was digested with the restriction enzymes Eco RI and Bam HI, gel purified using a Qiaex II resin as described above.

25 The excised and restriction digested zsig37 DNA was subcloned into plasmid CEE/pZP9 which had been cut with Eco RI and Bam HI. The zsig37CEE/pZP9 expression vector uses the native zsig37 signal peptide, and the Glu-Glu epitope (SEQ ID NO:26) is attached at the C-terminus
30 as a purification aid. Plasmid CEE/pZP9 (deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, ATCC No. 98668) is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, multiple restriction sites for
35 insertion of coding sequences, a sequence encoding the Glu-Glu tag (SEQ ID NO:26), a stop codon and a human growth hormone terminator. The plasmid also has an *E.*

coli origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

5 For the N- and C-tagged constructs, about 30 ng of the restriction digested inserts and 50 ng of the corresponding vectors were ligated at room temperature for 4 hours. One microliter of each ligation reaction was independently electroporated into DH10B competent cells
10 (GIBCO BRL, Gaithersburg, MD) according to manufacturer's direction and plated onto LB plates containing 50 mg/ml ampicillin, and incubated overnight.

Colonies were screened by PCR as described above. For zsig37NEE/pZP9 and zsig37CEE/pZP9 screens the
15 primers were ZC13006 (SEQ ID NO:29) and ZC13007 (SEQ ID NO:20). The PCR reaction was incubated at 94°C for 2.5 minutes, and then run for 25 cycles of 94°C for 10 seconds, 58°C for 20 seconds and 72°C for 1 minute. A 5 minute extension at 72°C followed. The insert sequence of
20 positive clones, 1013 bp for zsig37NEE and a 950 bp fragment for zsig37CEE were verified by sequence analysis. A large scale plasmid preparation was done using a QIAGEN® Maxi prep kit (Qiagen) according to manufacturer's instructions.

25

Example 5

Transfection and Expression of zsig37NEE and CEE Polypeptides

30

BHK 570 cells (ATCC No. CRL-10314) were plated in 10 cm tissue culture dishes and allowed to grow to approximately 50 to 70% confluency overnight at 37°C, 5% CO₂, in DMEM/FBS media (DMEM, Gibco/BRL High Glucose,
35 (Gibco BRL, Gaithersburg, MD), 5% fetal bovine serum (Hyclone, Logan, UT), 2 µM L-glutamine (JRH Biosciences, Lenexa, KS), 1 µM sodium pyruvate (Gibco BRL)). The cells

were then transfected with the plasmid zsig37NEE/pZP9 (N-terminal Glu-Glu tag) or zsig37CEE/pZP9 (C-terminal Glu-Glu tag), using LipofectamineTM (Gibco BRL), in serum free (SF) media formulation (DMEM, Gibco/BRL High Glucose, (Gibco BRL, Gaithersburg, MD), 2 mM L-glutamine, 2 mM sodium pyruvate, 10 ug/ml transferrin, 5 µg/ml insulin, 10 µg/ml fetuin and 2 ng/ml selenium). Sixteen micrograms of zsig37NEE/pZP9 and 16 µg of zsig37CEE/pZP9 were separately diluted into 15 ml tubes to a total final volume of 640 µl SF media. In separate tubes, 35 µl of LipofectamineTM (Gibco BRL) was mixed with 605 µl of SF medium. The LipofectamineTM mix was added to the DNA mix and allowed to incubate approximately 30 minutes at room temperature. Five milliliters of SF media was added to the DNA:LipofectamineTM mixture. The cells were rinsed once with 5 ml of SF media, aspirated, and the DNA:LipofectamineTM mixture was added. The cells were incubated at 37°C for five hours, then 6.4 ml of DMEM/10% FBS, 1% PSN media was added to the plate. The plate was incubated at 37°C overnight and the DNA:LipofectamineTM mixture was replaced with fresh FBS/DMEM media the next day. On day 2 post-transfection, the cells were split into the selection media (ESTEP #1 with 1 µM MTX) in 150 mm plates at 1:50, 1:100 and 1:200. The plates were refed at day 5 post-transfection with fresh selection media.

Screening colonies

Approximately 10-12 days post-transfection, one 150 mm culture dish of methotrexate resistant colonies was chosen from each transfection, the media aspirated, the plates washed with 10 ml serum-free ESTEP 2 media (668.7g/50L DMEM (Gibco), 5.5 g/50L pyruvic acid, sodium salt 96% (Mallinckrodt), 185.0 g/50L NaHCO₃ (Mallinckrodt), 5.0 mg/ml, 25 ml/50L insulin, 10.0 mg/ml and 25 ml/50 L transferrin). The wash media was aspirated and replaced with 5 ml serum-free ESTEP 2. Sterile Teflon mesh (Spectrum Medical Industries, Los Angeles, CA) pre-soaked

in serum-free ESTEP 2 was then placed over the cells. A sterile nitrocellulose filter pre-soaked in serum-free ESTEP 2 was then placed over the mesh. Orientation marks on the nitrocellulose were transferred to the culture dish. The plates were then incubated for 5-6 hours in a 37°C, 5% CO₂ incubator. Following incubation, the filter was removed, and the media aspirated and replaced with DMEM/5% FBS, 1X PSN (Gibco BRL) media. The filter was then placed into a sealable bag containing 50 ml buffer (25 mM Tris, 25 mM glycine, 5 mM β-mercaptoethanol) and incubated in a 65°C water bath for 10 minutes. The filters were blocked in 10% nonfat dry milk/Western A buffer (Western A: 50mM Tris pH 7.4, 5 mM EDTA, 0.05% NP-40, 150 mM NaCl and 0.25% gelatin) for 15 minutes at room temperature on a rotating shaker. The filter was then incubated with an anti-Glu-Glu antibody-HRP conjugate at a 1:1000 dilution in 2.5% nonfat dry milk/Western A buffer (Western A: 50mM Tris pH 7.4, 5 mM EDTA, 0.05% NP-40, 150 mM NaCl and 0.25% gelatin) overnight at 4°C on a rotating shaker. The filter was then washed three times at room temperature in PBS plus 0.1% Tween 20, 5-15 minutes per wash. The filter was developed with ECL reagent (Amersham Corp., Arlington Heights, IL) according the manufacturer's directions and exposed to film (Hyperfilm ECL, Amersham) for approximately 5 minutes.

The film was aligned with the plate containing the colonies. Using the film as a guide, suitable colonies were selected. Sterile, 3 mm colonizing discs (PGC Scientific Corp., Frederick, MD) were soaked in trypsin, and placed on the colonies. Twelve colonies for each construct were transferred into 200 µl of selection medium in a 96 well plate. A series of seven, two-fold dilutions were carried out for each colony. The cells were grown for one week at 37°C at which time the wells which received the lowest dilution of cells which are now at the optimum density were selected, trypsinized and transferred to a 12 well plate containing selection media. The 150 mm culture

dish was also trypsinized and the remainder of the cells were pooled and subjected to western analysis and mycoplasma testing. The pool was frozen for storage.

The clones were expanded directly from the 12 well plate into two T-75 flasks. One flask was kept to continue cell growth, the second flask was grown in serum-free ESTEP 2 which was harvested for Western Blot analysis. Clones of each of the expression constructs, based on Western blot analysis, were selected, pooled and transferred to large scale culture.

Example 7
Large Scale Mammalian Expression of zsig37CEE

One T-162 flask, containing confluent cells expressing zsig37CEE and one containing zsig37NEE obtained from the expression procedure described above, were expanded into four T-162 flasks each. One of the four resulting flasks was used to freeze down four cryovials, and the other three flasks were used to generate a Nunc cell factory.

The cells from the three T-162 flasks of zsig37CEE and zsig37NEE were used to independently seed two Nunc cell factories (10 layers, commercially available from VWR). Briefly, the cells from the T-162 flasks described above were detached using trypsin, pooled, and added to 1.5 liters ESTEP1 media (668.7g/50L DMEM (Gibco), 5.5 g/50L pyruvic acid, sodium salt 96% (Mallinckrodt), 185.0 g/50L NaHCO₃ (Mallinckrodt), 5.0 mg/ml and 25 ml/50L insulin (JRH Biosciences), 10.0 mg/ml and 25 ml/50L transferrin (JRH Biosciences), 2.5L/50L fetal bovine serum (characterized) (Hyclone), 1 μ M MTX, with pH adjusted to 7.05 +/-0.05) prewarmed to 37°C. The media containing the cells was then poured into the Nunc cell factories via a funnel. The cell factories were placed in a 37°C/5.0% CO₂ incubator.

At 80-100% confluence, a visual contamination test (phenol red color change) was performed on the

contents of the Nunc cell factories. Since no contamination was observed, supernatant from the confluent factories was poured into a small harvest container, sampled and discarded. The adherent cells were then
5 washed once with 400 ml PBS. To detach the cells from the factories, 100 mls of trypsin was added to each and removed and the cells were then incubated for 5 to 10 minutes in the residual trypsin. The cells were collected following two, 200 ml washes with ESTEP1 media. To each
10 of ten ESTEP1 media-containing bottles (1.5 liters each, at 37°C) was added 40 mls of collected cells. One 1.5 liter bottle was then used to fill one Nunc factory. Each cell factory was placed in a 37°C/5.0% CO₂ incubator.

At 80-90% confluence, a visual contamination
15 test (phenol red color change) was performed on the Nunc cell factories. Since no contamination was observed, supernatant from the confluent factories was poured into a small harvest container, sampled and discarded. Cells were then washed once with 400 ml PBS. 1.5 liters of
20 ESTEP2 media (668.7g/50L DMEM (Gibco), 5.5 g/50L pyruvic acid, sodium salt 96% (Mallinckrodt), 185.0 g/50L NaHCO₃ (Mallinckrodt), 5.0 mg/ml, 25 ml/50L insulin, 10.0 mg/ml and 25 ml/50L transferrin) was added to each Nunc cell factory. The cell factories were incubated at 37°C/5.0%
25 CO₂.

At approximately 48 hours a visual contamination test (phenol red color change) was performed on the Nunc cell factories. Supernatant from each factory was poured into small harvest containers. Fresh serum-free media
30 (1.5 liters) was poured into each Nunc cell factory, and the factories were incubated at 37°C/5.0% CO₂. One ml of supernatant harvest for each construct was transferred to a microscope slide, and subjected to microscopic analysis for contamination. The contents of the small harvest
35 containers for each construct were pooled and immediately filtered. A second harvest was then performed, substantially as described above at 48 hours and the cell

factories were discarded thereafter. An aseptically assembled filter train apparatus was used for aseptic filtration of the harvest supernatant (conditioned media). Assembly was as follows: tubing was wire-tied to an Opti-Cap filter (Millipore Corp., Bedford, MA) and a Gelman Supercap 50 filter (Gelman Sciences, Ann Arbor, MI). The Supercap 50 filter was also attached to a sterile capped container located in a hood; tubing located upstream of the Millipore Opti-cap filter was inserted into a peristaltic pump; and the free end of the tubing was placed in the large harvest container. The peristaltic pump was run between 200 and 300 rpm, until all of the conditioned media passed through the 0.22 μ m final filter into a sterile collection container. The filtrate was placed in a 4°C cold room pending purification. The media was concentrated 10X with a Millipore 5 kDa cut off concentrator (Millipore Corp., Bedford, MA) according to manufacturer's direction and subjected to Western Blot analysis using an anti-FLAG tag antibody (Kodak).

20

Zsig37CEE:

5 T-162 Flasks = 0.12 mg/L, 38 kDa;
1 Factory, FBS = 0.12 mg/L, 38 kDa;
10 Factories, FBS = 0.12 mg/L, 38 kDa;
10 Factories (#1), SF = 1.2 mg/L, 38 kDa; and
10 Factories (#2), SF = 3.56 mg/L, 38 kDa

25

Zsig37NEE:

5 T-162 Flasks = 0.137 mg/L, 35 kDa;
1 Factory, FBS = 0.137 mg/L, 35 kDa;
10 Factories, FBS = 0.137 mg/L, 35 kDa;
10 Factories (#1), SF = 1.37 mg/L, 35 kDa; and
10 Factories (#2), SF = 4.11 mg/L, 35 kDa.

30

35

Example 7
Purification of zsig37 NEE and zsig37 CEE

Unless otherwise noted, all operations were carried out at 4°C. The following procedure was used for purifying zsig37 containing N-terminal or C-terminal Glu-Glu (EE) tags. A total of 25 liters of conditioned media from baby hamster kidney (BHK) cells was sequentially sterile filtered through a 4 inch, 0.2 mM Millipore (Bedford, MA) OptiCap capsule filter and a 0.2 mM Gelman (Ann Arbor, MI) Supercap 50. The material was then concentrated to about 1.3 liters using a Millipore ProFlux A30 tangential flow concentrator fitted with a 3000 kDa cutoff Amicon (Bedford, MA) S10Y3 membrane. The concentrated material was again sterile-filtered with the Gelman filter as described above. A mixture of protease inhibitors was added to the concentrated conditioned media to final concentrations of 2.5 mM ethylenediaminetetraacetic acid (EDTA, Sigma Chemical Co. St. Louis, MO), 0.001 mM leupeptin (Boehringer-Mannheim, Indianapolis, IN), 0.001 mM pepstatin (Boehringer-Mannheim) and 0.4 mM Pefabloc (Boehringer-Mannheim). A 25.0 ml sample of anti-EE Sepharose, prepared as described below, was added to the sample for batch adsorption and the mixture was gently agitated on a Wheaton (Millville, NJ) roller culture apparatus for 18.0 h at 4°C.

The mixture was then poured into a 5.0 x 20.0 cm Econo-Column (Bio-Rad, Laboratories, Hercules, CA) and the gel was washed with 30 column volumes of phosphate buffered saline (PBS). The unretained flow-through fraction was discarded. Once the absorbance of the effluent at 280 nM was less than 0.05, flow through the column was reduced to zero and the anti-EE Sepharose gel was washed batch-wise with 2.0 column volumes of PBS containing 0.4 mg/ml of EE peptide (AnaSpec, San Jose, CA). The peptide used has the sequence Glu-Tyr-Met-Pro-Val-Asp, SEQ ID NO:31). After 1.0 h at 4°C, flow was resumed and the eluted protein was collected. This fraction was referred to as the peptide elution. The

anti-EE Sepharose gel was then washed with 2.0 column volumes of 0.1 M glycine, pH 2.5, and the glycine wash was collected separately. The pH of the glycine-eluted fraction was adjusted to 7.0 by the addition of a small
5 volume of 10X PBS and stored at 4°C for future analysis if needed.

The peptide elution was concentrated to 5.0 ml using a 15,000 molecular weight cutoff membrane concentrator (Millipore, Bedford, MA) according to the
10 manufacturer's instructions. The concentrated peptide elution was separated from free peptide by chromatography on a 1.5 x 50 cm Sephadex G-50 (Pharmacia, Piscataway, NJ) column equilibrated in PBS at a flow rate of 1.0 ml/min using a BioCad Sprint HPLC (PerSeptive BioSystems,
15 Framingham, MA). Two-ml fractions were collected and the absorbance at 280 nM was monitored. The first peak of material absorbing at 280 nM and eluting near the void volume of the column was collected. This fraction was pure zsig37 NEE or zsig37 CEE. The pure material was
20 concentrated as described above, analyzed by SDS-PAGE and Western blotting with anti-EE antibodies, and samples were taken for amino acid analysis and N-terminal sequencing. The remainder of the sample was aliquoted, and stored at -80°C according to our standard procedures.

25 Electrophoresis of zsig37 NEE on SDS-PAGE gels in the absence of reducing agents, showed one major Coomassie Blue-stained band of apparent molecular weight 39,000 and several minor components of molecular weights between 60,000 and 116,000. All of the bands showed cross
30 reactivity with anti-EE antibodies on Western blots. In the presence of reducing agent, the only band observed was the 39,000 kDa protein, and its Coomassie Blue staining intensity was increased. This band also showed cross-reactivity with the anti-EE antibody on Western blots.

35 For zsig37 CEE, electrophoresis on SDS-PAGE gels in the absence of reducing agents showed one major Coomassie Blue-stained band of apparent molecular weight

39,000 and several minor components of molecular weights between 60,000 and 116,000. On Western blots, only bands of apparent molecular weights 150,000, 116,000, and 60,000 showed cross-reactivity with the anti-EE antibody. In the presence of reducing agents, only the Coomassie Blue-stained band at 39,000 kDa was observed and this material showed cross-reactivity with the anti-EE antibody on Western blots. Under these conditions, a small amount of cross-reactive material was also seen at 150,000 kDa.

10

Preparation of anti-EE Sepharose

A 100 ml bed volume of protein G-Sepharose (Pharmacia, Piscataway, NJ) was washed 3 times with 100 ml of PBS containing 0.02% sodium azide using a 500 ml Nalgene 0.45 micron filter unit. The gel was washed with 6.0 volumes of 200 mM triethanolamine, pH 8.2 (TEA, Sigma, St. Louis, MO). and an equal volume of EE antibody solution containing 900 mg of antibody was added. After an overnight incubation at 4°C, unbound antibody was removed by washing the resin with 5 volumes of 200 mM TEA as described above. The resin was resuspended in 2 volumes of TEA, transferred to a suitable container, and dimethylpimilimidate-2HCl (Pierce, Rockford, IL), dissolved in TEA, was added to a final concentration of 36 mg/ml of gel. The gel was rocked at room temperature for 45 min and the liquid was removed using the filter unit as described above. Nonspecific sites on the gel were then blocked by incubating for 10 min at room temperature with 5 volumes of 20 mM ethanolamine in 200 mM TEA. The gel was washed with 5 volumes of PBS containing 0.02% sodium azide and stored in this solution at 4°C.

35

Example 8 Adhesion and Proliferation Assays

The ability of zsig37 to stimulate adhesion and spreading of TF-1 cells was assayed as follows. A series

of dilutions were prepared from C-terminal Glu-Glu-tagged zsig37, from 10 to 0.0625 µg/ml, in either PBS or ELISA coating buffer (0.1 M NaCO₃) and each was plated into a 96 well plate (Costar, Pleasanton, CA) at 100 µl/well. The plates were incubated at 37°C, 5% CO₂ for 2 hours. The plates were then washed 3X with RPMI/10% FBS (RPMI 1640, 2 mM L-glutamine, 110 µg/ml sodium pyruvate, PSN and 10% heat inactivated fetal bovine serum) and allowed to block for 15 minutes.

TF-1 cells (derived from acute myeloid leukemia cells) were resuspended in RPMI/10% FBS and plated into at 10,000 cells/well into the zsig37CEE-coated 96 well plates at a final volume of 120 µl/well. The plate was incubated at 37°C under 5% CO₂ for 2 hours. The plates were then washed 3X with PBS and 200 µl/well growth media (RPMI/10% FBS, 5ng/ml GM-CSF) was added. The cells were microscopically inspected before and after the wash.

A dye incorporation assay was also used to quantitatively measure the number of adherent cells based on a colorimetric change and an increase in fluorescent signal. Alamar BlueTM (AccuMed, Chicago, IL) was added to the 96 well plates and the cells were incubated at 37°C under 5% CO₂ overnight. The plates were then scanned using a fluorometer with excitation wavelength of 544 nm and emission wavelength of 590 nm. There were more adherent cells on the zsig37CEE-PBS coated plates than on the zsig37CEE-0.1 M NaCO₃ coated plates. Addition of soluble zsig37 did not block adhesion of cells to the bound zsig37.

A second assay was done using TF-1, DA-1 (an IL-3 dependent cell line derived from the lymph node of a mouse with a B-cell lymphoma by outgrowth in IL-3 media (provided by Dr. Kenneth Kaushansky, University of Washington, Seattle, WA)), pre-B (p53^{-/-} mouse marrow cells, IL-7 dependent, B220⁺, Thy1 low, Sca-1⁺), and A7BaF-3 cell lines as described above at 5,000 cells/well. BHK cells were also plated at 500 cells/well. Zsig37

enhanced the growth of A7-BaF-3 cells and slightly inhibited growth of DA-1 cells.

Example 9

Mouse Ortholog Sequence

5 The novel human zsig37 polypeptide-encoding polynucleotides of the present invention were used to screen a mouse EST database for homologous mouse sequences. A single EST sequence was discovered and
10 predicted to the human zsig37 sequence. To identify the corresponding cDNA, a clone considered likely to contain the entire coding sequence was used for sequencing. Using an Invitrogen S.N.A.P.TM Miniprep kit (Invitrogen Corp.) according to manufacturer's instructions a 5 ml overnight
15 culture in LB + 50 µg/ml ampicillin was prepared. The template was sequenced on an ABIPRISMTM model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT) using the ABI PRISMTM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.) according to manufacturer's
20 instructions. Oligonucleotides ZC694 (SEQ ID NO:6), ZC6768 (SEQ ID NO:32), ZC18297 (SEQ ID NO:33), ZC18298 (SEQ ID NO:34), ZC18402 (SEQ ID NO:35), ZC18403 (SEQ ID NO:36), ZC18456 (SEQ ID NO:37), ZC18457 (SEQ ID NO:38), ZC18560 (SEQ ID NO:39), ZC18561 (SEQ ID NO:40), ZC18687
25 (SEQ ID NO:41) and ZC18688 (SEQ ID NO:42) were used to complete the sequence from the clone. Sequencing reactions were carried out in a Hybaid OmniGene Temperature Cycling System (National Labnet Co., Woodbridge, NY). SEQUENCHERTM 3.1 sequence analysis
30 software (Gene Codes Corporation, Ann Arbor, MI) was used for data analysis. The resulting 2559 bp sequence is disclosed in SEQ ID NO:43 and the deduced amino acid sequence in SEQ ID NO:44. Alignment with the human zsig37 nucleotide sequence (SEQ ID NO:1) shows 77% identity at
35 the nucleotide level. The putative amino acid sequence (SEQ ID NO:44) has 77% identity with the human polypeptide sequence (SEQ ID NO:2).

Example 10
Cell Based Assays

5 Zsig37 polypeptides were assayed in a high throughput, *in vitro* assay to identify substances that selectively activate cellular responses in immortalized osteoblast cell lines. A mature osteoblast cell line derived from p53-/- (deficient) mice, CCC4, that is
10 transfected with a plasmid containing an inducible serum response element (SRE) driving the expression of luciferase was used in the assay. These cells also express endogenous PTH, PDGF and bFGF receptors. The stimulation of the SRE and thus the expression of
15 luciferase in the CCC4 cells indicates that the chemical entity is likely to stimulate mitogenesis in osteoblasts.

 CCC4 lines were trypsinized and adjusted to 5×10^4 cells/ml in plating medium (alpha-MEM, 1% heat inactivated fetal bovine serum, 1 mM Na pyruvate and 2 mM
20 L- glutamate) and plated (200 μ l/well) into Dynatech Microlite opaque white microtiter plates (Dynatech, Chantilly, VA) and incubated overnight at 37°C. 5% CO₂. The growth medium was then aspirated and replaced with 50 μ l/well assay medium (F-12 HAM, 0.5% bovine serum albumin,
25 20 mM HEPES, 1 mM Na pyruvate and 2 mM L-glutamate). Serial dilutions of zsig37 were made in assay medium (0.29-1000 ng/ml final assay concentration) and added to the wells. Zsig37 samples were assayed in triplicate. Serum (negative) and bFGF (positive) controls were also
30 used. Final concentration of bFGF was 3 ng/ml. Controls were assayed in quadruplicates. The plates were incubated for 4 hours at 37°C, 5% CO₂. The assay medium was then aspirated and the plates were rinsed once with PBS. To each well was then added 25 μ l of lysis buffer
35 (Luciferase Assay Reagent, E1501, Promega Corp., Madison, WI). The plates were incubated for 15 minutes at room temperature. Fifty microliters/well of luciferase

substrate (Luciferase Assay Reagent, E1501, Promega Corp.) was added and the Luciferase activity was detected using a Labsystems LUMINOSKAN[®] at 2 second/well following a 1 second delay. The average basal (uninduced) signal was
5 subtracted from all readings which are expressed in Table 5 as a percentage of the maximal induction produced by 3 ng/ml bFGF.

Zsig37 stimulates the expression of luciferase in this assay indicating that they stimulate osteoblasts.
10 Zsig37 stimulates at 73 to 75% maximal at 1000 ng/ml.

A counter part growth factor mimetic assay was performed to determine if zsig37 is acting as a growth factor mimetic, particularly tyrosine kinase receptor ligands PDGF, bFGF and EGF (Insulin-R negative). A clonal
15 cell line derived from Swiss 3T3 mice, Swiss 3T3, that is transfected with a plasmid containing an inducible serum response element (SRE) driving the expression of luciferase was used in the assay. These cells also express endogenous PMA, EGF and bFGF receptors. The
20 stimulation of the SRE and thus the expression of luciferase in the Swiss 3T3 cells indicates that the chemical entity is likely mimics the PDGF, bFGF and EGF growth factor activity.

Swiss 3T3 cells were trypsinized and adjusted to
25 5×10^4 cells/ml in plating medium, plated and incubated as described above. The growth medium was then aspirated and replaced with 50 μ l/well assay medium (F-12 HAM, 0.5% bovine serum albumin, 20 mM HEPES). Serial dilutions of zsig37 were made in assay medium (0.29-1000 ng/ml final
30 assay concentration) and added to the wells. Zsig37 samples were assayed in triplicate. A serum (negative) and bFGF (positive) control to promote cell proliferation were also used. Final concentration of bFGF was 3 ng/ml. Controls were assayed in quadruplicates. The plates were
35 incubated for 5 hours at 37°C, 5% CO₂. The assay medium was then aspirated and the plates were rinsed once with PBS. To each well was then added 25 μ l of lysis buffer

(Luciferase Assay Reagent, E1501, Promega Corp., Madison, WI). The plates were incubated for 15 minutes at room temperature. Forty microliters/well of luciferase substrate (Luciferase Assay Reagent, E1501, Promega Corp.) was added and the Luciferase activity was detected using a Labsystems LUMINOSKAN[®] at 2 second/well following a 1 second delay. The average basal (uninduced) signal was subtracted from all readings which are expressed as a percentage of the maximal induction produced by 3 ng/ml bFGF. A five hour treatment of this cell line with bFGF, DDGF, EGF or PMA leads to a 25-50 fold induction of SRE-luciferase expression.

Zsig37 does not appear to stimulate the expression of luciferase in this assay. Zsig37 stimulates at 0.2 to 0.1% maximal at 1000 ng/ml.

Example 10

In vivo Administration of zsig37 Via Adenoviral Delivery

Twenty four male and 24 female C57B16/J mice, approximately 12 weeks old (Jackson Labs, Bar Harbor, ME) were weighed, body temperature was measured and food intake monitored daily for four days prior to injection (days -4 to -1). On day 0, the mice were divided into three groups and received 0.1 ml virus (AdV-empty 1.8×10^{11} virus particles/0.1 ml or AdV-zsig37-CEE 5×10^{11} virus particles/0.1 ml) by intravenous tail vein injection, or no injection at all. Injection should result in infection of the host's liver and expression of virally delivered gene should commence within 24 hours and continue for 1 to 4 weeks. Three groups of mice were tested. Group 1, untreated, n=8 each male and female. Group 2, AdV-Empty (empty virus), n=8 each male and female. Group 3, AdV-zsig37 CEE, n=8 each male and female.

The animals' body temperatures, weights and the weight of food ingested was monitored during the three week study. No difference was found between the groups.

On day 21 the female mice were euthanized and sacrificed by cervical dislocation, and on day 22 the males were. The animals were exsanguinated and tissues harvested for necropsy.

5 The standard serum chemistry panel was done at the time of sacrifice. Liver, kidney and metabolic parameters were all within normal ranges. There was, however a difference between the zsig37 treatment group and the empty virus treated group. The zsig37 animals had
10 a higher average lipemic index than the empty virus controls. The difference was not significant, however further investigation was warranted. Total free fatty acids were assayed on the remaining serum from each animal. A statistically significant difference in serum
15 Free Fatty Acid levels was seen between male mice ($p=0.0379$) receiving empty virus and those receiving zsig37 encoding virus; the zsig37 mice had higher levels. A difference, though not statistically significant, was also seen in females ($p=0.3357$). Liver, spleen, kidney,
20 thymus, heart and brain were weighed after removal. No difference was found between the treatment groups. Histopathological analysis of these tissues and bone marrow revealed no difference between the treatment groups.

25 To confirm the above results a second screen was done as above with the following modifications. Three groups; a) untreated and fasted, b) AdV-null and fasted, c) AdV-zsig37-CEE and fasted, containing 20 C57B16/J, 10 each male and female, were tested. The mice were fasted
30 overnight and 100 μ l serum was collected to establish a basal level for the following parameters: fasting glucose, TP, alkaline phosphatase, cholesterol, triglycerides, free fatty acids and insulin. Body weights were taken three times a week. On day 0, mice were injected into the
35 lateral tail vein with 0.1 ml of the appropriate virus solution. Blood was collected on day 17 following an overnight fast. After 3 weeks the mice were sacrificed

and all blood collected. A portion of the blood was mixed with EDTA to look at CBC's and the remainder will be re-assayed and screened as described above. Organs were collected and the carcass saved for histopathology.

5

Example 11
Vasodilatation of Aortic Rings

10 The effect of zsig37 on vasodilatation of aortic rings was measured according to the procedures of (Dainty et al., J. Pharmacol. 100:767, 1990 and Rhee et al., Neurotox. 16: 179, 1995). Briefly, aortic rings 4 mm in length were taken from 4 month old Sprague Dawley rats and
15 placed in modified Krebs solution (118.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂·2H₂O, 24.8 mM NaHCO₃ and 10 mM glucose). The rings were then attached to an isometric force transducer (Radnoti Inc., Monrovia, CA) and the data recorded with a Ponemah
20 physiology platform (Gould Instrument systems, Inc., Valley View, OH) and placed in a 10 ml tissue bath oxygenated (95% O₂, 5% CO₂) modified Krebs solution. The tissues were adjusted to 1 gram resting tension and allowed to stabilize for one hour before testing. The
25 rings were tested by 5μl additions of 1X10⁻⁷ M norepinephrin (Sigma Co., St. Louis, MO) to a final concentration of about 1X10⁻⁹ M and Carbachol, a muscarinic acetylcholine agonist (Sigma Co.) at 2X1⁻⁷ M final, to test the integrity of the rings. After each test
30 the rings were washed three times with fresh buffer, 5 minutes between washes and allowed to rest one hour. To test for vasodilatation, the rings were contracted to two grams and allowed to stabilize for fifteen minutes. Zsig37 was then added to 1, 2 or 3 of the 4 baths, without
35 flushing, and tension on the rings was recorded and compared to the control rings. The rings were then tested for contraction with norepinephrin as described above. Rings were tested at 323, 162, and 81 ng/ml zsig37 but a

dose response could not be determined. In order to evaluate the statistical significance of the data, a contingency test was done on all the zsig37 and control rings using dilation as a determinant. Of 10 of the 12
5 rings tested with zsig37 vasodilated as did 2 of the 7 controls. The Fisher exact P value is 0.045. It was concluded that zsig37 induces vasodilatation in norepinephrin contracted aortic rings.

10

Example 12Binding of zsig37 to matrix proteins

An ELISA (Enzyme-linked Immunosorbant Assay) was used to measure binding of zsig37 to various matrix
15 proteins and complement Clq. The matrix proteins used were Bovine Collagen Type I (Becton Dickinson, Lincoln Park, NJ) laminin, vitronectin, fibronectin, human collagen Types II, III, IV, V, VI (Chemicon International, Temecula, CA). BSA V (Sigma Co.) was used as a negative
20 control. Just prior to use, the proteins were diluted in 2 X PBS (Phosphate Buffered Saline, Sigma Co.) to 100 µg/ml and adjusted to pH 7.2 with 0.1 N NaOH. Each protein sample was quadruplicate plated (100 µl/well) into a 96 well plate. The plate was allowed to dry overnight
25 in a laminar flow hood and washed 3 times with 400 µl of 5 mg/ml BSA in 1 X PBS and blotted dry. Zsig37 was FITC labeled according to manufacturer's instruction (Pierce, Rockford, IL). Into each well was added 100 µl of 1.8 µg/ml zsig37-FITC in 5% BSA, PBS. The plates were
30 incubated for 1.5 hours at room temperature then washed 3 time with 5% BSA, PBS. To each well was then added 100 µl of 1:400 mouse anti-FITC/Biotin (Sigma Co.). The plate was incubated 1.5 hours at room temperature and washed 3 times with 5% BSA, PBS. The plate was then incubated with
35 100 µl of 1:1000 streptavidin/HRP (Amersham, Piscataway, NJ) for 1 hour and washed 3 times with 5% BSA, PBS. The plate was then developed using Supersignal® Ultra (Pierce, Rockford, IL) according to manufacturer's instruction.

After reacting for 1 minute, surplus liquid was removed from the plate by inverting the plate and patting dry. The plate was exposed to X-ray film (Kodak, Rochester, New York).

5 The results of this screen indicate that only fibronectin and the collagens I, II, III, V and VI bind significantly to zsig37-FITC. Such binding was not seen with laminin, vitronectin, collagen IV or the BSA control.

10 Example 13
Specificity of Zsig37 Binding to Collagen Type VI

The ELISA assay for binding as described in Example 12 was modified to quantitatively evaluate binding. Zsig37-FITC, in a range of 0.4 to 4 $\mu\text{g/ml}$, was bound to 10 μg of collagen type VI (Chemicon International) as described in above. The luminescence from the Supersignal[®] reagent was read on a Wallac 1420 plate reader (Wallac, Gaithersburg MD) and the intensity used as a quantitative measure of the zsig37-FITC bound to the ELISA plate.

The binding of zsig37 to collagen type VI fits a typical hyperbolic binding curve (Figure 3a). The bound Zsig37-FITC plated at $0.4\mu\text{g/ml}$ can be competed off the
25 collagen by the addition of unlabeled Zsig37 in a range of 0.8 to $8\mu\text{g/ml}$ (Figure 3b). These data would indicate that binding is specific for domains on collagen type VI and is concentration dependent.

Example 14

Zsig37 Binding to complement C1q

Zsig37-FITC at 0.2 $\mu\text{g/ml}$ was shown to bind to complement C1q (Sigma Co.) at 0.1 to 10 $\mu\text{g/ml}$ by the method described above in Example 13, (Figure 4). The amount of binding is concentration dependent and saturable.

Example 15
Complement Inhibition by Zsig37

5 Complement assays were performed in 96 well
round bottom plates. Gelatin Veronal buffer containing
magnesium and calcium (141 mM NaCl, 1.8 mM sodium
barbitol, 3.1 mM Barbituric acid, 0.1% bovine gelatin, 0.5
mM MgCl₂ and 0.15 mM CaCl₂) was used for all serum and
10 inhibitor dilutions as well as erythrocyte suspensions.
Fifty microliters of standardized human Complement serum
(Sigma Co.), diluted 1/37.5 (for a final dilution of
1/150) was added to each well. The inhibitor was added in
triplicate, 50 µl/well. The serum and inhibitor were
15 incubated for thirty minutes at room temperature. The
assay was initiated by the addition of 100 µl of 2 X 10⁸/ml
unsensitized sheep erythrocytes (Colorado Serum Co.,
Denver, CO), sensitized sheep erythrocytes, sensitized
using the Hemolysin manufacturer's protocol (BioWhittaker
20 Inc., Walkersville, MD) and rabbit erythrocytes containing
16 mM EGTA, and 4 mM Mg⁺⁺. A human serum dilution series
from 1/50 to 1/400 was also plated as an activity control.
Erythrocytes, lysed with distilled water and diluted to
100, 75, 50, 25, and 12.5 percent lysis, were used to
25 quantify Complement percent lysis. The plate was sealed
and incubated at 37°C for 1 hour with mixing every 15
minutes. The reaction was stopped by the addition of 220
mM EDTA, 20 µl/well and the plates centrifuged at 1500 X G
for 10 minutes. One hundred microliters of supernatant was
30 removed from each well and transferred to a 96 well flat
bottom plate for analysis. The plate was read at 415 nM
and percent lysis was calculated.

 Zsig37 was effective in inhibiting the
classical pathway with both sensitized and unsensitized
35 sheep erythrocytes (Figure 5). There was no apparent
inhibition of the alternate pathway tested with rabbit
erythrocytes and EGTA. The mechanism of inhibition is

undetermined but because Clq binds zsig37, C1 is the most likely target.

Example 16

5 Inhibition by Zsig37 of platelet collagen activation

Blood was drawn from healthy volunteers into tubes containing sodium citrate, maintained at room temperature and used within four hours of drawing. Whole
10 blood was analyzed for platelet activation using a Chrono-Log 560A Whole Blood Lumi-Aggregometer (Chrono-Log Corp., Haverton, PA) according to manufacturer's instructions. For each test point, 500 μ l of blood was added to a reaction tube containing a stir bar and 500 μ l of isotonic
15 saline containing zsig37 at concentrations from 0 to 20 μ g/ml. The mixture was incubated for four minutes followed by platelet activation initiated by the addition of 5 μ l of 1 mg/ml cross-linked collagen (Chrono-Log Corp.) to the blood/zsig37 mixture. Inhibition of activation by
20 ADP (final concentration 10 μ M), and thrombin (final concentration 1U/ml) was tested in a similar way.

Inhibition of collagen-mediated platelet activation by zsig37 shows a dose dependent relationship between 5 and 20 μ g/ml (Figure 6a). The inhibition is
25 selective for collagen activation and has no effect on activation stimulated by ADP or thrombin (Figure 6b). Collagen activation was not inhibited by another complement Clq related protein zsig39 (co-pending US Patent Application 09/140,804).

30

Example 17

Stimulation of SK5 Fibroblast growth by Zsig37

Human fibronectin (25 ug/ml, GIBCO BRL,
35 Gaithersburg, MD) was plated into 96 well plates (Costar, Pleasanton, CA) at 100 μ l/well and allowed to dry in a laminar hood overnight. Human SK5 fibroblasts in DMEM (Gibco) containing 10% Fetal Bovine Serum - low endotoxin (Hyclone, Logan, UT) were plated at 5000 cells/well into

the fibronectin-coated plates and incubated at 37°C, 5% CO₂ for 2 to 3 days. The number of cells per plate was adjusted to achieve non-confluence. Cells were then washed twice with serum-free DMEM hi glucose (Gibco) and serum starved by growing in serum-free DMEM hi glucose for 24 hours. Zsig37 was added to the wells in triplicate, at concentrations of 312.2 ng/ml to 10,000 ng/ml in 100 µl DMEM. The cells were then incubated for 48 hours at 37°C, 5% CO₂. Cell proliferation was tested by adding 15 µl MTT dye solution (CellTiter96™ kit, Promega) to each well. The plate was incubated 4 hours at 37°C, 5% CO₂ and the reaction was stopped with Solubilization/Stop solution (CellTiter96™ kit, Promega) according to the manufacturer's instructions. The plate was incubated for 1 hour to solubilize formazan crystals and the absorbance was measured at 570 nm with a reference at 650 nm using an ELISA plate reader.

The results (Figure 7) show a dose dependent increase in SK5 fibroblast number over the range of zsig37 concentrations tested. These concentrations were within the range of values seen for the mitogenic effects of the fibrinogen b chain (Gray, et al, Am J. Respir. Cell Mol. Biol. 12, 684,1995 and Gray, et al, J. Biol. Chem. 270, 26602, 1995) and for fibroblast cell adhesion to plated C1q (Bordin, and Ghebrehiwet, J. Immun. 145:2520, 1990) both of which are believed to interact with cell surface calreticulin.

Example 18 Zsig37 Anti-Sera Production

Rabbit polyclonal anti-sera was prepared by immunizing two female New Zealand white rabbits with zsig37-CEE purified from BHK cells. The protein was conjugated to the carrier protein keyhole limpet hemocyanin (KLH) with gluteraldehyde. The rabbits were each given an initial intraperitoneal (ip) injection of 200 µg of peptide in Complete Freund's Adjuvant followed

by booster ip injections of 100 µg peptide in Incomplete Freund's Adjuvant every three weeks. Seven to ten days after the administration of the second booster injection, the animals were bled and the serum was collected. The
5 animals were then boosted and bled every three weeks.

Example 19

Detection of FITC Tagged Zsig37 Protein Binding in Tissues

10 FITC tagged Zsig37 protein binding in tissues was detected as follows:

Paraffin embedded and sectioned human tissues or mouse embryos on slides were obtained either from commercial sources (i.e. DAKO Corporation, Carpinteria,
15 CA; BioGenex, San Ramon, CA; Novagen, Madison, WI; and Biomeda, Foster City, CA) or in house. The human tissue sections included adrenal gland, brain, heart, small intestine, large intestine, kidney, liver, lung, ovary, pancreas, prostate, spleen, stomach, testis, thyroid, and
20 uterus. The mouse embryo sections were from the 16 day stage.

The tissue sections were dewaxed using standard conditions of 3 x 5 minutes in xylene, 4 minutes in 100% ethanol (EtOH), 3 minutes in 100% EtOH, and 2 minutes in
25 95% EtOH. The tissue sections were then subjected to a 20 minute antigen retrieval process at 94°C according to the manufactures instructions (DAKO Corporation), followed by a 20 minute 0.01% Pepsin/0.2 N HCl digest. The tissue sections were rinsed twice in dH₂O and once in PBS/0.05%
30 Tween 20 (Sigma, St. Louis, MO) buffer and then blocked for 45 minutes with 1 X PBS/5% BSA/5% nonfat dry milk (Carnation, Los Angeles, CA). This was followed by an avidin/biotin blocking step done according to the manufacturers instructions (Vector Laboratories, Inc.,
35 Burlingame, CA). The tissue sections were washed 3 times in 1 X PBS/0.05% Tween 20 buffer and then incubated with appropriate concentration of FITC tagged Zsig37 protein in PBS/5% BSA for 45-60 minutes. After washing the tissue

sections 3 times in 1 X PBS/0.05% Tween 20, they were incubated with a 1:400 dilution of anti-FITC (mo) MAb Biotin conjugate (Sigma) for 30-60 minutes, washed 3 times in PBS/0.05% Tween 20 and then incubated for 30-60 minutes with a 1:500 dilution of Streptavidin-FITC (NEN Life Science Products, Boston, MA) followed by 2 washes in 1 X PBS/0.05% Tween 20 buffer and 1 wash in 1 X PBS without Tween 20. The tissues sections were then mounted with an antifade medium containing 0.5 μ g/ml propidium iodide as counterstain.

Significant binding was seen to vessel walls and fine fibrous connective type tissues such as collagenous matrix in the majority of the human tissues and embryo sections studied.

15

Example 20
Zsig37 in the Rabbit Carotid Artery Injury Model

Zsig37 was administered in a modified rabbit carotid artery injury model (Folts et al., Circulation 79:116-24, 1989 and Golino et al., Thrombosis and Haemostasis 67:302-5, 1992) to determine the degree of protection offered in preventing vascular occlusion following a crush injury.

Thirty four male New Zealand White rabbits, approximately 3-6 months old (R&R Rabbitry, Stanwood, WA) were into two groups. Fifteen rabbits received doses of zsig37 ranging from 2-13.5 μ g/kg and 19 control rabbit were injected with PBS or equivalent amounts of PBS or zsig39, another adipocyte complement related protein (WO99/10492). The rabbits were anesthetized with ketamine (50 mg/kg, IM) and maintained on halothane inhalation anesthesia for the duration of the study. The hair was shaved from the ears and neck and an angiocatheter was placed in the marginal ear vein for IV support. A midline incision was made in the neck and the carotid artery was accessed. Approximately 5 cm of the common carotid artery

proximal to the internal/external bifurcation was exposed via blunt dissection away from the surrounding tissue and any visible side branches were cauterized. A flow probe (Transonic Systems, Inc., Ithaca NY) was placed distal to the anticipated injury site and a baseline blood flow was established. A 2.5-3.0 cm section of the vessel was then isolated from circulation using atraumatic vascular clamps. Following removal of the blood from the vessel segment, 0.4 ml of zsig37 in 0.9% sodium chloride or 0.04 ml 0.9% sodium chloride as a control, was injected into the empty vessel segment using a 30G needle. The vessel was left undisturbed for a 5 minute pre-injury treatment. A 1.0 cm crush injury was then inflicted into the center of the vessel segment using a guarded hemostat and left undisturbed for 10 minutes. The vessel clamps were then removed and blood flow reestablished. Blood flow was monitored continuously for 60 minutes after which time the rabbits were euthanized and the vessel excised for histological analysis.

No dose dependency was seen at these concentrations. A meta analysis of all zsig37 doses resulted in significant increase in time patent when compared to controls in an unpaired t-test ($P=0.019$).

The mean percent time patent for the combined groups of negative control animals, as determined from blood flow tracings, was 13.5% with a standard error of $\pm 1.7\%$. The mean percent time patent for the combined zsig37 treated groups of animals, as determined from blood flow tracings, was 37.2% with a standard error of $\pm 10.3\%$.

In a second series of experiments, fluoresceinated zsig37 was used in the injured carotid artery model. Male, New Zealand White rabbits were anesthetized as above. Via an incision in the neck, the carotid artery was exposed and approximately 5 cm of the vessel isolated from the surrounding consecutive tissue. Blood was evacuated from the isolated segment and

atraumatic vascular clips were applied. Approximately 0.05 ml of fluoreceinated zsig37 (concentration 100 µg/ml) was injected into the isolated segment to completely fill the vessel using a 30g needle. After an exposure period of 5 minutes, the vessel was injured and the exposure continued for another 110 minutes before the clips were removed and blood flow reestablished. The animals were euthanized as described above at 1, 10, and 60 minutes post-reestablishment of blood flow and the vessels collected and formalin fixed for histological evaluation.

Labeled zsig37 preferentially bound to receptors in the media of the injured vessels. Labeled zsig37 did not bind to areas of the vessel that were uninjured. The time of blood flow prior to vessel collection does not appear to effect the amount of zsig37 that remains bound to the tissue, i.e., there was no difference in the amount of labeled zsig37 bound to the tissues in the 1 minute vs. The 60 minute collection time point. This may indicate that zsig37 tightly binds to the injured vessel and is not washed off by the reestablished blood flow.

The effect of zsig37 on blood flow dynamics following vascular injury in a rabbit iliac artery crush injury/stenosis model was also evaluated. Young adult male New Zealand White rabbits were anesthetized as described above. Via an abdominal incision, the aorto-iliac bifurcation was exposed and each iliac freed of surrounding tissues and the main branches ligated. Each iliac was instrumented with an ultrasound flow probe to monitor blood flow through the vessel. Based on blood flow data, one iliac was selected to be used for the injury and the other was catheterized for delivery of the test sample. Rabbits were divided into dose groups of 6 animals/group. Test sample doses containing zsig37 increased in half-log increments from 3-1000 µg/kg over the selected infusion period. The test samples infusion was initiated followed by creation of a critical stenosis that reduced blood flow through the vessel by

approximately 50%. After creation of the stenosis and a period of blood flow stabilization, the vessel was injured by crushing the vessel between the jaws of a smooth needle holder. The infusion was continued post-injury for a set period of time, 10-20 minutes. Blood flow through the injured vessel was monitored for 60 minutes post-injury. The animals were euthanized at the conclusion of the study period. The lower section of the abdominal aorta and each iliac were collected and formalin fixed for histological evaluation.

Blood flow parameters determined from the flow tracings, included mean flow post-stenosis, mean flow post-injury, and time the vessel remained patent. This data suggests there is a tendency for zsig37 to promote increased patency time with increased dose up to 300 µg/kg/ over a 60 minute period.

Example 21
Relaxation of serotonin-induced rat aortic ring
contractions

Male, Sprague-Dawley rats, approximately 3 months of age, were lightly anesthetized with CO₂ and then decapitated. The thoracic aorta was then rapidly removed and placed in a modified Krebs-Henseleit buffer (NaCl, 118.2 mM; KCl, 4.6 mM; CaCl₂, 2.5 mM; MgSO₄, 1.2 mM; NaHCO₃, 24.8 mM; KH₂PO₄, 1.2 mM; and glucose, 10.0 mM). From each rat, four 2-3 mm aortic ring sections were cut after discarding the rough end of the aorta. In some experiments the endothelium was denuded, prior to cutting ring sections, by rubbing the lumen of the aorta along a 21 gauge needle. Denudation of the endothelium was verified by the addition of the acetylcholine analogue, carbachol, prior to determining zsig27 concentration-dependent responses. In the absence of the endothelium, carbachol does not vasorelax constricted vascular ring sections.

The rings were fixed and connected to force displacement transducers in oxygenated (95% O₂, 5% CO₂),

jacketed, glass organ baths kept at 30 °C in modified
Kreb's-Henseleit buffer, pH 7.4. Resting tension was set
at 1 gm, and continually re-adjusted to 1 gm over a 1 hour
incubation period. Fresh oxygenated modified Kreb's-
5 Henseleit buffer was added to the baths every fifteen
minutes during the resting incubation period. At the end
of the 1 hour incubation, the ring sections were
contracted by the addition of 10 μ M serotonin. After
maximum contraction had been reached, approximately 15-20
10 minutes after the addition of the serotonin, cumulative
concentration response curves for zsig37 were constructed.
Zsig37 was added to 5 ml baths in volumes from 5 up to 150
 μ ls, for final concentrations ranging from 1 ng/ml up to
40 μ g/ml. Viability of the ring sections was verified at
15 the end of the concentration response by the addition of
forskolin (2.5 μ M or 25 μ M) or nitroglycerin (22 μ M).

Addition of zsig37 induced a concentration-
dependent vasorelaxation of serotonin-contracted rat
aortic sections with and without an intact endothelium.
20 Relaxation in response of zsig37 was first observed at
concentrations above 100 ng/ml. Relaxation was observed
approximately 30-60 seconds after the additions of each
zsig37 concentration to the bath, and relaxation responses
plateaued within 3-5 minutes after the addition of zsig37.
25 The character of the relaxation response to zsig37
indicates that the vasorelaxation is a receptor-second
messenger mediated event. Additionally, the ability of
zsig37 to vasorelax endothelium-denuded aortic sections
indicates that zsig37 acts directly on the smooth muscle
30 cells to elicit the vasorelaxant response.

Example 22

Identification of cells expressing zsig37 receptor using in situ hybridization

35 Specific human tissues were isolated and
screened for zsig37 expression by *in situ* hybridization.
Various human tissues prepared, sectioned and subjected to

in situ hybridization included aorta, heart, lymph node, placenta, prostate, salivary gland, skin, and testis. The tissues were fixed in 10% buffered formalin (Surgipath, Richmond, IL), and embedded in paraplast X-tra (Oxford Scientific, St. Louis, MO), and sectioned at 5 μ m with a Reichart-Jung 2050 microtome (Leica Instruments GmbH, Nussloch, Germany). Tissues were sectioned at 4 to 8 microns. Tissues were prepared using a standard protocol ("Development of nono-isopotic *in situ* hybridization", Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences, Research Park Triangle, NC). Briefly, tissue sections were deparaffinized with HistoClear (National Diagnostics, Atlanta, GA) and then dehydrated with ethanol. Next the sections were digested with Proteinase K (50 μ g/ml) (Boehringer Mannheim, Indianapolis, IN) at 37°C for 2 to 20 minutes. This step was followed by acetylation and re-hydration of the tissues.

Three *in situ* probes generated by PCR were designed against the human zsig37 sequence. Two sets of oligonucleotide primers were designed to generate probes for separate regions of the zsig37 cDNA: (1) Oligonucleotide ZC23,689 (SEQ ID NO:45) and ZC23,694 (SEQ ID NO:46) were used to generate a 414 bp probe for zsig37; (2) Zc23,703 (SEQ ID NO:47) and ZC23,697 (SEQ ID NO:48) were used to generate a 896 bp probe for zsig37; (3) ZC24,441 (SEQ ID NO:49) and ZC24,442 (SEQ ID NO:50) were used to generate a 290 bp probe for zsig37. The antisense oligo from each set also contained the working sequence for the T7 RNA polymerase promoter to allow for easy transcription of antisense RNA probes from these products. The PCR products were purified by Qiagen spin columns (Qiagen, Inc., Chatsworth, CA) followed by phenol/chloroform extraction and ethanol precipitation. Probes were subsequently labeled with digoxigenin

(Boehringer) or biotin (Boehringer) using an *in vitro* transcription system (Promega Corp., Madison, WI) according to the manufacturer's instructions.

In situ hybridization was performed with a digoxigenin- or biotin-labeled zsig37 probe as described above. The probe was added to the slides at a concentration of 1 to 5 pmol/ml for 12 to 16 hours at 50-60 °C. Slides were subsequently washed in 2X SSC and 0.1X SSC at 50-55°C. Slides were subsequently washed in 2X SSC and 0.1X SSC at 50-55°C. The signals were amplified using tyramide signal amplification (TSA *in situ* indirect kit, NEN, Boston, MA) and visualized with a Vector Red substrate kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. The slides were then counter-stained with hematoxylin (Vector Laboratories).

Positive signals were seen in the human aorta, heart, prostate, salivary gland, and testis. The positive-staining cells appeared to be endothelial cells of small diameter vessels in the adventitia surrounding the aorta, mesothelial cells overlying the epicardium, acinar cells of the salivary gland and scattered mononuclear cells, trophoblasts of the placenta, epithelial cells of the prostate and stratified epithelium of the seminiferous tubules of testis.

Example 23 SEC-MALLS Analysis of Zsig37

Zsig37 contains a N-terminal collagen-like domain as well as a C-terminal globular region having homology to the tumor necrosis factor family and like other such proteins, zsig37 is expected to multimerize. Purified zsig37 analyzed using an ESI-ion trap mass spectrometer (Finnigan Matt, San Jose, CA) indicated the presence of species approximating trimers and 9mers, which was an unexpected result when compared to other homologous proteins. Peptide mapping of zsig37 using LC-MS/MS on an

ESI-ion trap mass spectrometer (Finnigan Matt) revealed that several cysteine residues were modified with an S-cysteinylyl group. It may be that modification of key cysteine residues in the zsig37 protein during
5 fermentation with free cysteine in the media is preventing proper oligomeric association of this molecule.

To learn more, a comparison of reduced and nonreduced zsig37 was made using a Biosep S-300 size exclusion column at 1.0 ml/minute (7.8 x 3000 mm;
10 Phenomenex, Torrance, CA) on a HP 1050 HPLC (Hewlett Packard, Heidleberg, Germany). The HP 1050 was coupled to light scattering and refractive index detectors, miniDAWN and Optilab DSP, (Waytt Technology, Santa Barbara, CA) for on-line SEC-MALLS.

15 One milligram of recombinant zsig37 (1.0 mg/ml) was added to TCEP at a 10:1 mol/mol ratio of TCEP to zsig37 and kept at room temperature for 70 minutes. Sixty microliters of the reduced zsig37 was injected for SEC-MALLS analysis and the remainder of the reduced zsig37 was
20 dialyzed in 0.5-3.0 ml Slide-A-Lyzer cassettes, 10K MWCO (Pierce, Rockford, IL) with stirring against PBS, pH 7.4 with three buffer changes as follows: 1 liter PBS at room temperature for 4 hours, 1 liter PBS at 4°C overnight, and 1 liter PBS at room temperature for 4 hours.

25 Following dialysis, oxidation was allowed to continue at 4 °C. The oxidation was moitored by SEC-MALLS analysis of aliquites taken at three time points, T=0 hours, T=24 hours, and T=96 hours. Molecular weight values were determined using the LS-RI, two-detector
30 method.

Analysis of the reduced, dialyzed recombinant zsig37 seems to initially indicate the formation of hexamers and 18mers as detected by SEC-MALLS which is more consistant with the oligomeric states observed in

homologs. These forms were also active in *in vitro* assays.

Example 24
Zsig37 Binding to Monocytes

5

CD14 positive monocytes were isolated from frozen peripheral blood aphaeresis product using a positive selection method with Miltenyi beads (Miltenyi Biotec Auburn, CA). Purified cells were greater than 80% CD14
10 positive via FACS staining. Cells were resuspended at 1×10^6 cells/ml in RPMI + 10% fetal bovine serum (FBS) and plated in 100 mm tissue culture dishes, 5 ml/plate. Recombinant human γ interferon was added at 100 ng/ml and the cells were incubated at 5% CO₂, 37°C for 48 hours.

15

The cells were removed from the plates by nonenzymatic methods using both EDTA and scraping, concentrated by centrifugation, resuspended in FACS staining buffer and aliquoted at 500,000 cells/tube for staining. Nonactivated cells were obtained by performing
20 another CD14 selection with the same apheresis product post 48 hours in γ interferon. The cells were incubated in varying concentrations of biotinylated zsig37 followed by Streptavidin PE. All blocking with "cold" zsig37 was done on ice for 30 minutes. Unbound protein was removed by
25 washing once in FACS buffer. Binding was quantitated using a FACS calibur instrument (Becton Dickinson, Lincoln Park, NJ) and expressed as a signal above secondary antibody only control. Monocyte activation was verified by approximately a 1 log increase in ICAM-1 expression in γ
30 interferon treated cells.

Zsig37 binding was detected in both activated and nonactivated monocytes, with an increase in zsig37 binding observed in γ interferon-treated cells. Binding was detected down to 1.5 μ g/ml, the lowest concentration
35 tested. At 15 μ g/ml, binding was approximately 4 fold

increased in activated cells. A slight (approximately 10%) reduction in binding is seen in activated cells only whe pretreated with 70 fold excess "cold" zsig37. Increases zsig37 binding in activated monocytes suggests
5 that the up-regulation of monocyte binding proteins/receptors for zsig37 by inflammatory cytokines. This could potentially result in zsig37 involvement in monocyte phagocytosis, microbial killing, and cellular cytotoxicity. Following the two days in culture, there
10 are macrophages present in the culture and zsig37 may be binding preferentially to this subset of cells. There are no good macrophage markers available to determine if this is occurring. Zsig37 also bound to a mouse monocyte/macrophage line, RAW 264.7 (ATCC No. CRL-2278),
15 indicating macrophage specificity.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the
20 spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

What is claimed is:

1. A method of promoting blood flow within the vasculature of a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; in a pharmaceutically acceptable vehicle; whereby said adipocyte complement related protein reduces thrombogenic and complement activity within said vasculature.

2. A method according to claim 1 wherein said adipocyte complement related protein comprises a polypeptide comprising a sequence of amino acid residues that is at least 75% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2, wherein said sequence comprises:

Gly-Xaa-Xaa or Gly-Xaa-Pro repeats forming a collagen domain, wherein Xaa is any amino acid, and a carboxy-terminal globular portion.

3. A method according to claim 2, wherein said polypeptide comprises a sequence of amino acid residues that is at least 90% identical in amino acid sequence to residues 22-281 of SEQ ID NO:2.

4. A method according to claim 2, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2.

5. A method according to claim 3, wherein any differences between said polypeptide and SEQ ID NO:2 are due to conservative amino acid substitutions.

6. A method according to claim 3, wherein said collagen domain consists of 13 Gly-Xaa-Xaa repeats and 1 Gly-Xaa-Pro repeat.

7. A method according to claim 3, wherein said globular domain consists of ten beta sheets.

8. A method according to claim 7, wherein said beta sheets are associated with amino acid residues correspondg to 147-151, 170-172, 178-181, 191-203, 207-214, 219-225, 227-239, 244-250, and 269-274 of SEQ ID NO:2.

9. A method according to claim 2, wherein said polypeptide comprises residues 1-281 of SEQ ID NO:2 or residues 1-281 of SEQ ID NO:44.

10. A method according to claim 2, wherein said polypeptide is complexed to a second polypeptide to form a oligomer.

11. A method according to claim 10, wherein said polypeptides are complexed by intermolecular disulfide bonds.

12. A method according to claim 11, wherein said oligomer is a trimer.

13. A method according to claim 11 wherein said oligomer is a hexamer.

14. A method according to claim 11 wherein said multmer is an 18mer.

15. A method according to claim 1, wherein said polypeptide reduces thrombogenic and complement activity by inhibition of the complement pathway and inhibition collagen-mediated platelet adhesion, activation or aggregation.

16. A method according to claim 1, wherein said polypeptide is administered prior to, during or following an acute vascular injury in said mammal.

17. A method according to claim 16, wherein said injury is due to vascular reconstruction.

18. A method according to claim 17, wherein said vascular reconstruction comprises angioplasty, coronary artery bypass graft, endarterectomy, microvascular repair or anastomosis of a vascular graft.

19. A method according to claim 18, wherein said injury is due to trauma, stroke or aneurysm.

20. A method of pacifying damaged collagenous tissues within a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; whereby said protein renders the damaged collagenous tissue inert towards complement activation, thrombotic activity or immune activation

21. A method according to claim 20, wherein said adipocyte complement related protein comprises a polypeptide comprising a sequence of amino acid residues that is at least 75% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2, wherein said sequence comprises:

Gly-Xaa-Xaa or Gly-Xaa-Pro repeats forming a collagen domain, wherein Xaa is any amino acid, and a carboxy-terminal globular portion.

22. A method according to claim 21, wherein said polypeptide comprises a sequence of amino acid residues that is at least 90% identical in amino acid sequence to residues 22-281 of SEQ ID NO:2.

23. A method according to claim 21, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2.

24. A method according to claim 22, wherein any differences between said polypeptide and SEQ ID NO:2 are due to conservative amino acid substitutions.

25. A method according to claim 22, wherein said collagen domain consists of 13 Gly-Xaa-Xaa repeats and 1 Gly-Xaa-Pro repeat.

26. A method according to claim 22, wherein said globular domain consists of ten beta sheets.

27. A method according to claim 26, wherein said beta sheets are associated with amino acid residues corresponding to 147-151, 170-172, 178-181, 191-203, 207-214, 219-225, 227-239, 244-250, and 269-274 of SEQ ID NO:2.

28. A method according to claim 22, wherein said polypeptide comprises residues 1-281 of SEQ ID NO:2 or residues 1-281 of SEQ ID NO:44.

29. A method according to claim 22, wherein said polypeptide is complexed to a second polypeptide to form a oligomer.

30. A method according to claim 29, wherein said polypeptides are complexed by intermolecular disulfide bonds.

31. A method according to claim 29, wherein said oligomer is a trimer.

32. A method according to claim 29 wherein said oligomer is a hexamer.

33. A method according to claim 29 wherein said multimer is an 18mer.

34. A method according to claim 20, wherein said damaged collagenous tissues are due to injury associated with ischemia and reperfusion.

35. A method according to the claim 34, wherein said injury comprises trauma injury ischemia, intestinal strangulation, or injury associated with pre- and post-establishment of blood flow.

36. A method according to claim 20, wherein said polypeptide is administered to a mammal suffering from cardiopulmonary bypass ischemia and recirculation, myocardial infarction, or post-trauma vasospasm.

37. A method according to claim 36, wherein said post-trauma vasospasm comprises stroke, percutaneous transluminal angioplasty, endarterectomy, accidental vascular trauma or surgical-induced vascular trauma.

38. A method of pacifying the surface of a prosthetic biomaterial for use in association with a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein;

whereby said polypeptide renders the surface of said prosthetic biomaterial inert towards complement activation, thrombotic activity or immune activation.

39. A method according to claim 38, wherein said adipocyte complement related protein comprises a polypeptide comprising a sequence of amino acid residues that is at least

75% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2, wherein said sequence comprises:

Gly-Xaa-Xaa or Gly-Xaa-Pro repeats forming a collagen domain, wherein Xaa is any amino acid, and a carboxy-terminal globular portion.

40. A method according to claim 39, wherein said polypeptide comprises a sequence of amino acid residues that is at least 90% identical in amino acid sequence to residues 22-281 of SEQ ID NO:2.

41. A method according to claim 39, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2.

42. A method according to claim 40, wherein any differences between said polypeptide and SEQ ID NO:2 are due to conservative amino acid substitutions.

43. A method according to claim 40, wherein said collagen domain consists of 13 Gly-Xaa-Xaa repeats and 1 Gly-Xaa-Pro repeat.

44. A method according to claim 40, wherein said globular domain consists of ten beta sheets.

45. A method according to claim 44, wherein said beta sheets are associated with amino acid residues corresponding to 147-151, 170-172, 178-181, 191-203, 207-214, 219-225, 227-239, 244-250, and 269-274 of SEQ ID NO:2.

46. A method according to claim 40, wherein said polypeptide comprises residues 1-281 of SEQ ID NO:2 or residues 1-281 of SEQ ID NO:44.

47. A method according to claim 39, wherein said polypeptide is complexed to a second polypeptide to form a oligomer.

48. A method according to claim 47, wherein said polypeptides are complexed by intermolecular disulfide bonds.

49. A method according to claim 47, wherein said oligomer is a trimer.

50. A method according to claim 47 wherein said oligomer is a hexamer.

51. A method according to claim 47 wherein said multimer is an 18mer.

52. A method of pacifying the surface of a prostatic biomaterial according to claim 38, wherein the surface of said prostatic biomaterial is coated with collagen or collagen fragments, gelatin, fibrin or fibronectin.

53. A method of mediating wound repair within a mammal comprising administering to said mammal a therapeutically effective amount of an adipocyte complement related protein; whereby said polypeptide enhances progression in wound healing.

54. A method according to claim 53, wherein said adipocyte complement related protein comprises a polypeptide comprising a sequence of amino acid residues that is at least 75% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2, wherein said sequence comprises:

Gly-Xaa-Xaa or Gly-Xaa-Pro repeats forming a collagen domain, wherein Xaa is any amino acid, and a carboxy-terminal globular portion.

55. A method according to claim 54, wherein said polypeptide comprises a sequence of amino acid residues that is at least 90% identical in amino acid sequence to residues 22-281 of SEQ ID NO:2.

56. A method according to claim 54, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical in amino acid sequence to residues 26-281 of SEQ ID NO:2.

57. A method according to claim 55, wherein any differences between said polypeptide and SEQ ID NO:2 are due to conservative amino acid substitutions.

58. A method according to claim 55, wherein said collagen domain consists of 13 Gly-Xaa-Xaa repeats and 1 Gly-Xaa-Pro repeat.

59. A method according to claim 55, wherein said globular domain consists of ten beta sheets.

60. A method according to claim 59, wherein said beta sheets are associated with amino acid residues corresponding to 147-151, 170-172, 178-181, 191-203, 207-214, 219-225, 227-239, 244-250, and 269-274 of SEQ ID NO:2.

61. A method according to claim 55, wherein said polypeptide comprises residues 1-281 of SEQ ID NO:2 or residues 1-281 of SEQ ID NO:44.

62. A method according to claim 55, wherein said polypeptide is complexed to a second polypeptide to form a oligomer.

63. A method according to claim 62, wherein said polypeptides are complexed by intermolecular disulfide bonds.

64. A method according to claim 62, wherein said oligomer is a trimer.

65. A method according to claim 62, wherein said oligomer is a hexamer.

66. A method according to claim 62, wherein said multimer is an 18mer.

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Fig. 1

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Fig. 2

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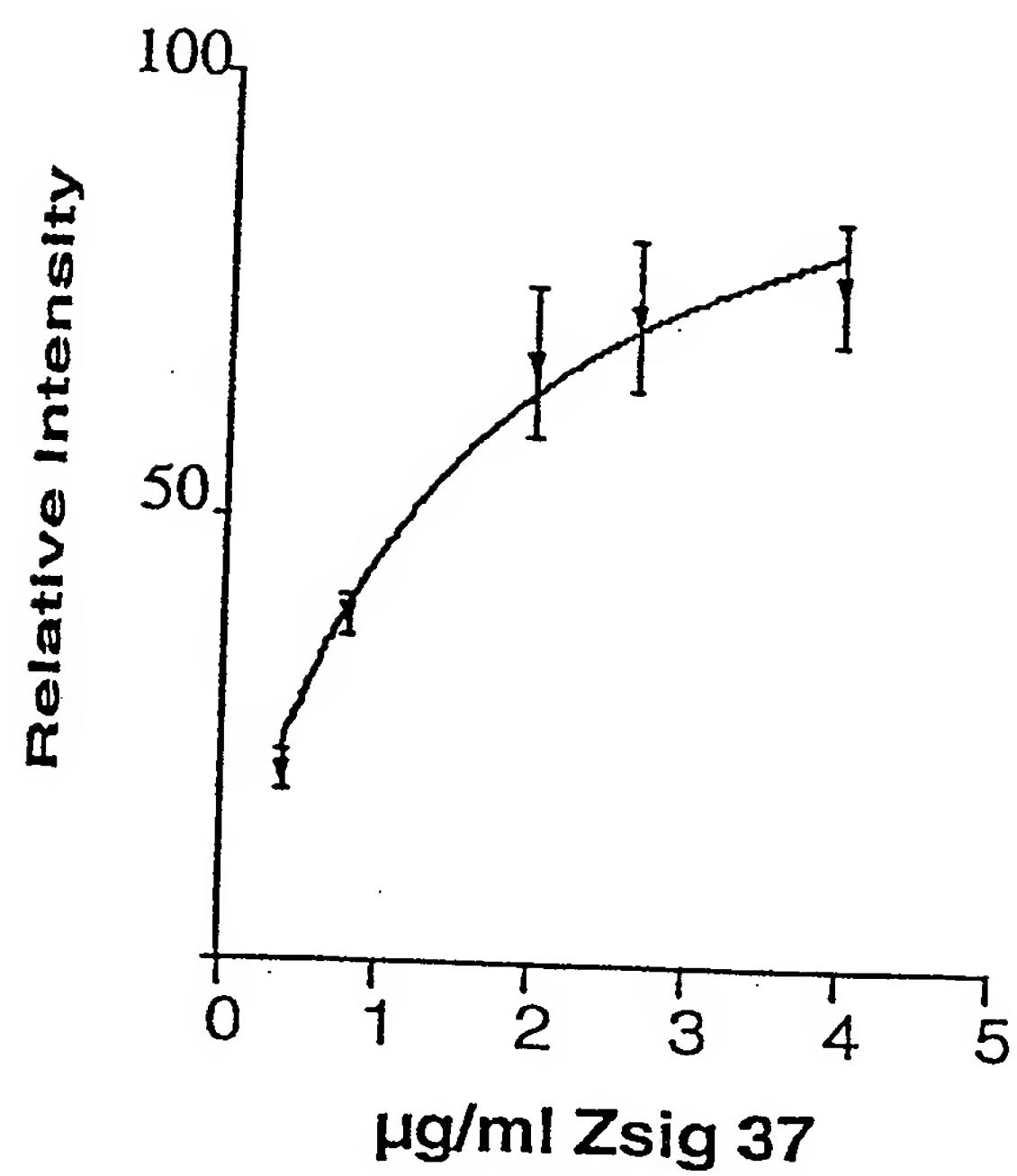


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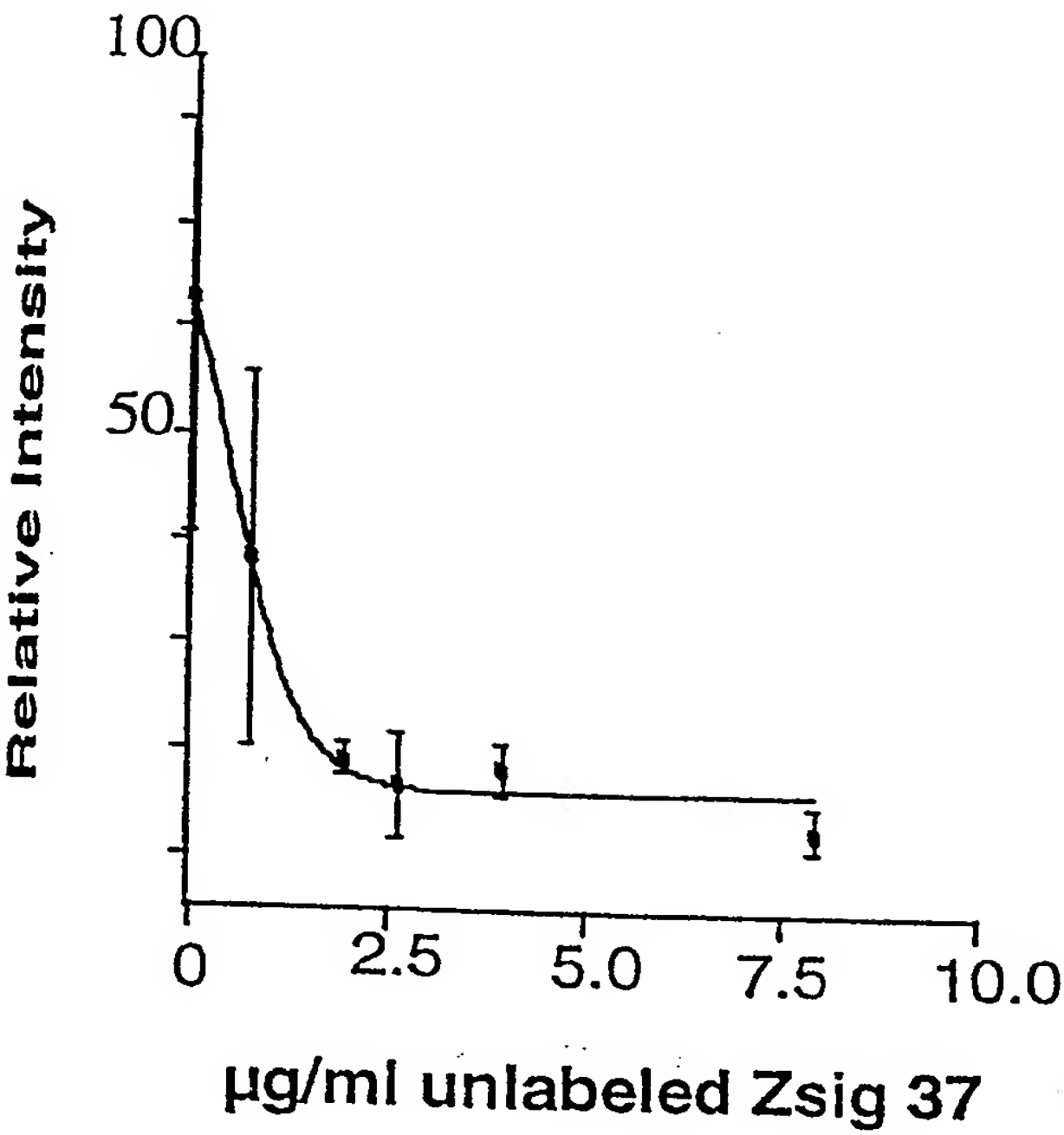


Fig. 3b

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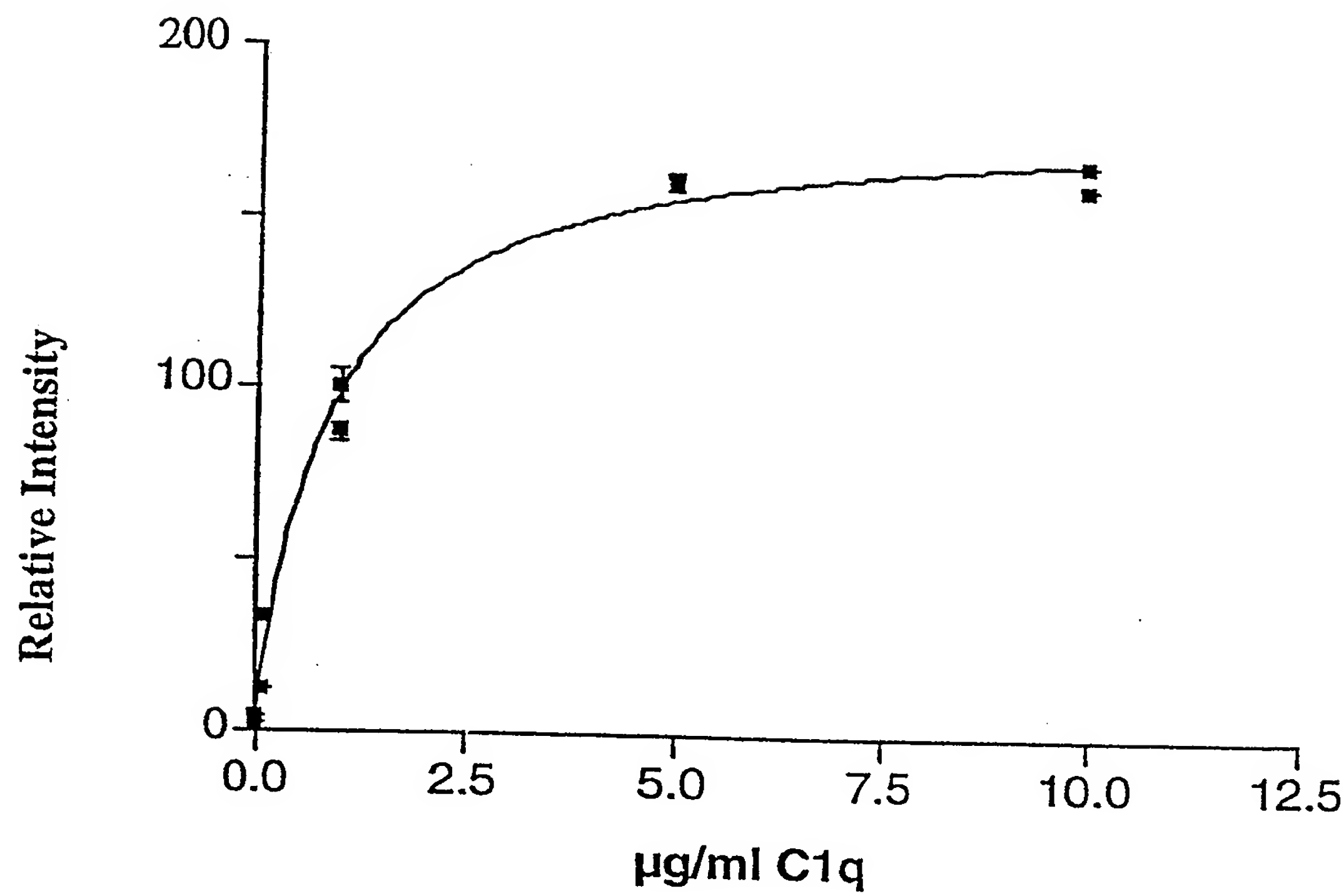


Fig. 4

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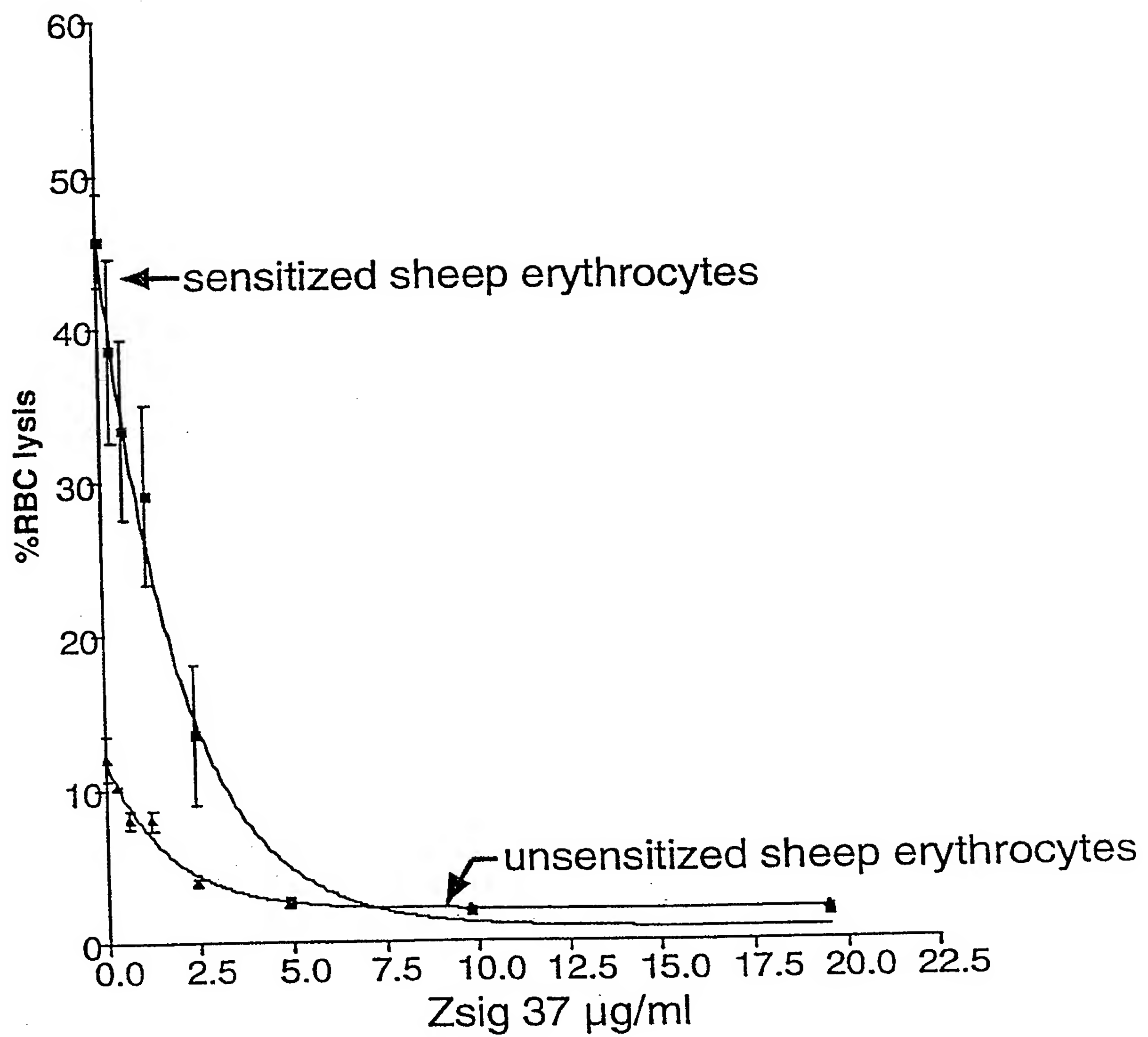


Fig. 5

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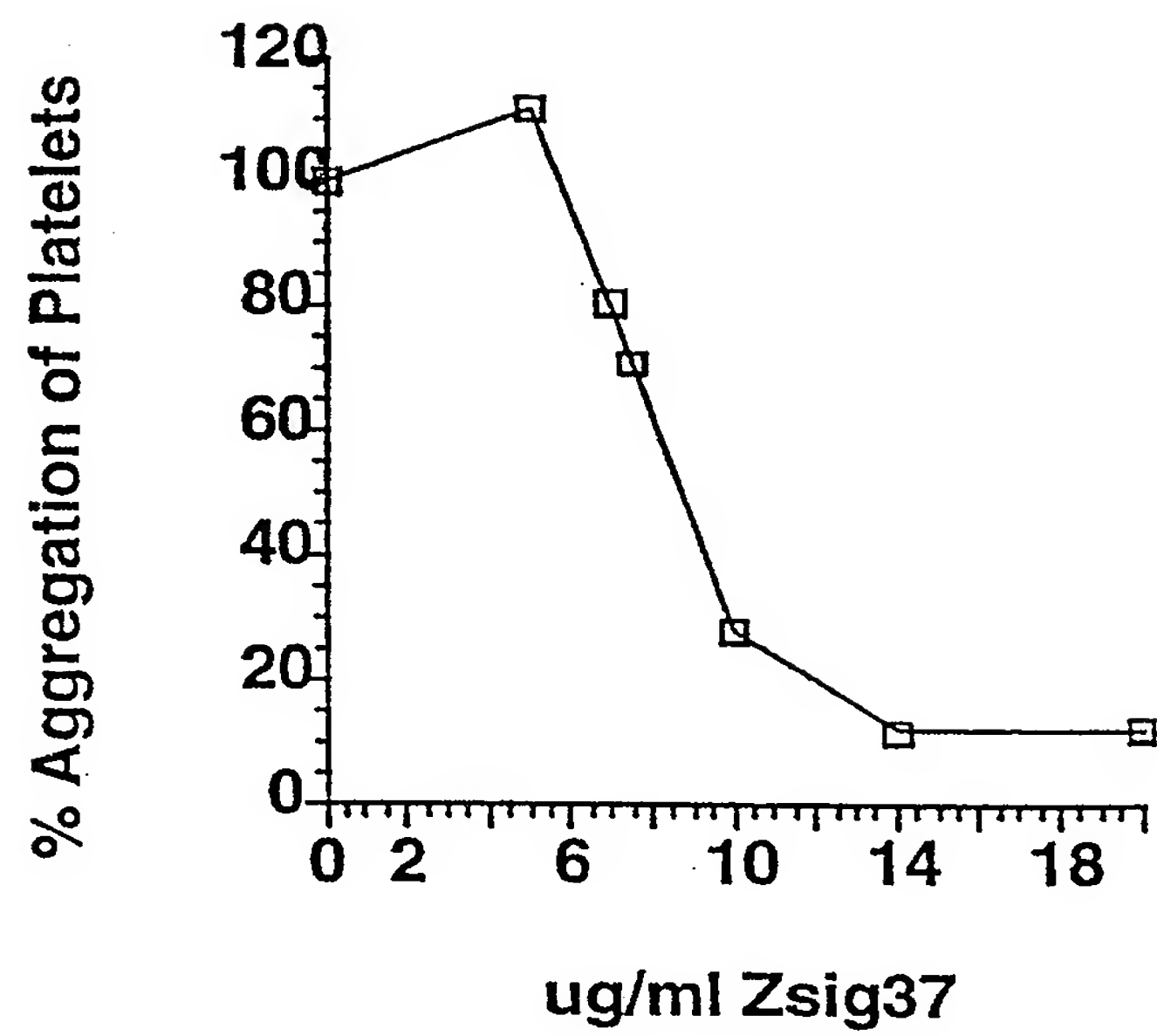


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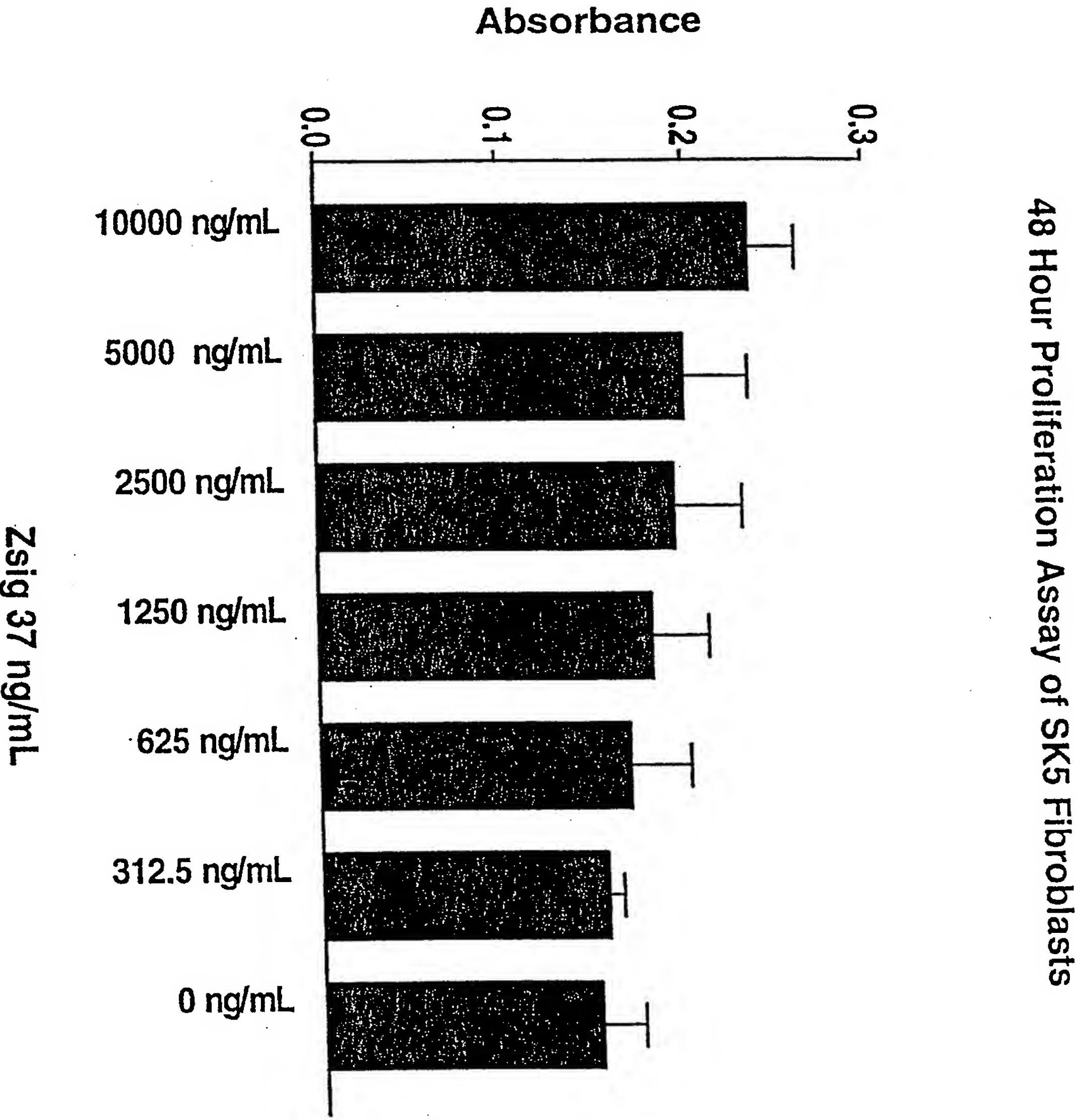


Fig. 7

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(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
24 August 2000 (24.08.2000)

PCT

(10) International Publication Number
WO 00/48625 A3

(51) International Patent Classification⁷: **A61K 38/17**,
A61P 9/00, 37/00

(21) International Application Number: PCT/US00/04161

(22) International Filing Date: 17 February 2000 (17.02.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/253,604 19 February 1999 (19.02.1999) US
09/444,794 22 November 1999 (22.11.1999) US

(71) Applicant: **ZYMOGENETICS, INC.** [US/US]; 1201
Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventors: **SHEPPARD, Paul, O.**; 13532 278th Drive
Northeast, Granite Falls, WA 98252 (US). **LASSER, Ger-
ald, W.**; 1611 S. Lake Stickney Drive, Lynnwood, WA
98037 (US). **BISHOP, Paul, D.**; 28425 S.E. 8th Street, Fall
City, WA 98024 (US).

(74) Agent: **LINGENFELTER, Susan, E.**; ZymoGenetics,
Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,
UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent
(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

(88) Date of publication of the international search report:
28 December 2000

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: INHIBITORS FOR USE IN HEMOSTASIS AND IMMUNE FUNCTION

(57) Abstract: The present invention relates to polynucleotide and polypeptide molecules for use as inhibitors in hemostasis and immune function. Such inhibitors are members of the family of proteins bearing a collagen-like domain and a globular domain. The inhibitors are useful for promoting blood flow in the vasculature by reducing thrombogenic and complement activity. The inhibitors are also useful for pacify collagenous surfaces and modulating wound healing.

WO 00/48625 A3



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/04161

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/17 A61P9/00 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, PAJ, WPI Data, MEDLINE, EMBASE, LIFESCIENCES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHERER PHILIPP E ET AL: "A novel serum protein similar to Clq, produced exclusively in adipocytes." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 45, 1995, pages 26746-26749, XP002148728 ISSN: 0021-9258 the whole document	1-66
A	SHAPIRO LAWRENCE ET AL: "The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor." CURRENT BIOLOGY, vol. 8, no. 6, 12 March 1998 (1998-03-12), pages 335-338, XP002148729 ISSN: 0960-9822 the whole document	1-66

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

28 September 2000

Date of mailing of the international search report

11/10/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/04161

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 869 330 A (SCHERER P.E. ET AL.) 9 February 1999 (1999-02-09) the whole document ---	1-66
A	WO 99 04000 A (ZYMOGENETICS) 28 January 1999 (1999-01-28) page 2 -----	1-66

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/04161

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5869330 A	09-02-1999	WO 9639429 A	12-12-1996
WO 9904000 A	28-01-1999	AU 8411398 A	10-02-1999
		EP 1002075 A	24-05-2000
		NO 20000215 A	17-03-2000